

Predictors of vaccine virus replication, immune response and clinical protection following oral rotavirus vaccination in Malawian children

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by

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DECLARATION

I declare that this thesis was composed by me, and that the work presented is my own, except where otherwise stated. It has not been submitted for any other degree or qualification.

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This project was funded by a Wellcome Trust Clinical PhD Fellowship (102464).

MY ROLE

I conducted and designed the cohort and case:control studies. My studies were integrated with the existing VacSurv diarrhoeal surveillance platform, led by Dr Naor Bar-Zeev, and with the RotaRITE Transmission Epidemiology study, led by Dr Aisleen Bennett. Standard operating procedures for recruitment and data collection for the RotaRITE studies were written by myself and Dr Bennett. Data collection forms for the case-control study were adapted from the VacSurv study. Laboratory standard operating procedures were written by myself and Dr Bennett with input from Prof Miren Iturriza Gomara and Dr Khuzwayo Jere.

Dr Bennett and I jointly managed the RotaRITE research nurses and field work team, who recruited patients and completed data collection. Data management, including writing data cleaning programmes, was performed by myself and Dr Bennett, assisted by Mr Richard Wachepa, Data Officer, and Mr Clemens Masesa, Senior Data Manager.

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ABSTRACT

Background

Current rotavirus vaccines are least effective in low-income, high-burden countries. Reduced vaccine response is likely to be multifactorial. The aim of this thesis was to determine whether passively-acquired maternal antibody levels, oral polio vaccine (OPV) response and histo-blood group antigen (HBGA) phenotype predict vaccine virus replication, immune response and clinical protection in Malawian infants following oral administration of the monovalent human rotavirus vaccine (RV1).

Methods

In a longitudinal cohort study, infants received two doses of RV1 at 6 and 10 weeks of age. Stool was collected on alternate days for 10 days post-vaccine to detect RV1 and OPV vaccine virus shedding by RT-PCR. Pre and post-vaccine serum rotavirus(RV)-specific IgA and IgG were determined by ELISA, with seroconversion defined as change from seronegative (RV-specific IgA<20 u/mL) to seropositive (RV-specific IgA>20U/mL) or >4x rise in concentration in infants seropositive at baseline. HBGA phenotype was determined by salivary ELISA and confirmed by FUT2 genotyping. Infants with detectable of A, B, or H antigens in saliva were defined as secretors. Infants with detectable Lewis a or b antigens in saliva were defined as Lewis positive and those with undetectable Lewis antigens as Lewis negative. In a separate cross-sectional case-control study, vaccinated infants <12 months with rotavirus gastroenteritis (RVGE) were compared to age-matched, vaccinated community and non-RVGE controls. Rotavirus detection and genotyping were confirmed by RT-PCR.

Results

Following rotavirus immunisation, 104/202 (52%) of infants had detectable vaccine virus shedding and 47/196 (24%) achieved RV-specific IgA seroconversion. Infants with the highest levels of maternal rotavirus-specific IgG antibody had reduced likelihood of vaccine virus shedding (RR 0.44, 95%CI 0.27-0.72, p=0.001) and lower RV-specific IgA response. Linear correlation between RV-specific IgG and vaccine response was weak, suggesting a threshold effect.

There was no correlation between quantitative rotavirus and OPV vaccine virus shedding, but some evidence of common patterns of OPV and RV1 response. Protective poliovirus type 3 neutralizing antibody titres at 6 weeks were associated with RV1 shedding in the 1st RV1 dose period (RR 2.24, 95%CI 1.25-4.0, p=0.007). OPV shedding in the 2nd RV1 dose period was associated with RV1 shedding (RR 2.0, 95%CI 1.0-3.8, p=0.04). All 14 infants who failed to attain protective serotype 3 poliovirus-specific neutralizing antibody titres also failed to attain rotavirus vaccine seroconversion.

There was no association observed between any HBGA phenotype and overall likelihood of vaccine virus shedding or seroconversion. In a sub-study of 186 infants, HBGA phenotype determined genotype-specific susceptibility to rotavirus infection: secretor phenotype was strongly associated with P[8] RVGE (OR 7.8, 95%CI 1.8-33.7, p=0.005) and P[4]RVGE (OR 5.8, 95%CI 1.3-25.2, p=0.02) and Lewis negative phenotype was associated with P[6] infection (OR 3.0, 85%CI 1.3-6.7, p=0.008). Comparing 119 RVGE cases to 119 age-matched community controls, non-secretor phenotype was associated with decreased risk of clinical rotavirus vaccine failure (OR 0.40, 95%CI 0.2-0.8, p=0.005).

RV-specific IgA >90U/mL at time of presentation was associated with a 75% decrease in the odds of clinical vaccine failure. Infants with vaccine failure mounted a robust convalescent RV-specific IgA response.

Conclusions

A threshold inhibitory effect of maternal antibody, together with the strong association of low RV-specific IgA and vaccine failure, suggests that booster dosing regimens could potentially improve RV1 effectiveness in Malawi. Further research is required to determine the optimal schedule. There was no evidence of direct competitive inhibition observed between OPV or RV1 vaccine virus shedding patterns. Common factors may predict response to both vaccines.

Contrary to our hypothesis, non-secretor infants were at decreased risk of clinical vaccine failure, due to relative protection against common rotavirus strains. HBGA phenotype is unlikely to contribute to reduced rotavirus vaccine effectiveness in Malawi. New non-P[8] based vaccines are therefore unlikely to confer additional benefit based on the HBGA hypothesis.

LIST OF ABBREVIATIONS

AIC	Akaike Information Criterion
bOPV	Bivalent oral poliovirus vaccine
BIC	Bayesian Information criterion
BRV-PV	Heat-stable, multivalent bovine reassortant rotavirus vaccine
CI	Confidence interval
Ct	Cycle threshold
CV	Co-efficient of variation
DNA	Deoxyribonucleic acid
EED	Environmental enteric dysfunction
EIA	Enzyme immuno-assay
ELISA	Enzyme-linked immuno-assay
ENS	Enteric nervous system
EPI	Extended programme of immunisation
FUT2	Fucosyltransferase 2
FUT3	Fucosyltransferase 3
G	Glycoprotein type
GI	Gastrointestinal
Gavi	Global Vaccine Alliance
GEMS	Global Enteric Multicenter Study
GMT	Geometric mean titre
GPS	Global positioning system
HBGA	Histo-blood group antigen
HIV	Human immunodeficiency virus
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPV	Inactivated parental poliovirus vaccine
Le^a	Lewis a
Le^b	Lewis b
LLR	Lanzhou lamb rotavirus vaccine
MAL-ED	The Etiology, Risk Factors, and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health Study
MUAC	Mid-upper arm circumference
mRNA	Messenger ribonucleic acid
NSP1-6	Non-structural protein 1-6
OD	Optical density
OPV	Oral poliovirus vaccine
OR	Odds ratio

ORV-116E	G9P[11] based neonatal rotavirus vaccine, ROTAVAC®
P	Protease-sensitive type
P2-VP8-P[8]	Parenteral, non-replicating P2-VP8-P[8] sub-unit vaccine
pIgR	Poly-immunoglobulin receptor
PROVIDE	Performance of Rotavirus and Oral Polio Vaccines in Developing Countries study
QECH	Queen Elizabeth Central Hospital
qRT-PCR	Quantitative real-time polymerase chain reaction
RIX4414	Precursor to RV1 vaccine
RoVi	Rotavirus vaccine immunogenicity study
RotaRITE	Rotavirus: Response to Immunisation and Transmission Epidemiology study
RNA	Ribonucleic acid
RR	Relative risk
RRV-TV	Rhesus reassortant tetravalent rotavirus vaccine, RotaShield®
RT-PCR	Real-time polymerase chain reaction
RV	Rotavirus
RV1	Monovalent human rotavirus vaccine, Rotarix®
RV5	Pentavalent human-bovine reassortant rotavirus vaccine, RotaTeq®
RV3-BB	G3P[6] based human neonatal rotavirus vaccine
RV GE	Rotavirus gastroenteritis
RV-NA	Rotavirus neutralizing antibodies
slgA	Secretory immunoglobulin A
TAC	Taqman array cards
tOPV	Trivalent oral poliovirus vaccine
U/mL	Units per millilitre
VacSurv	New Childhood Vaccines for Malawi surveillance study
VP1-7	Virus structural protein 1-7
VP8*	Subunit of VP4
WHO	World Health Organization

TABLE OF CONTENTS

Declaration.....	2
Funding	2
My Role	2
Acknowledgements.....	3
Abstract.....	5
List of Abbreviations	7
List of Figures	14
List of Tables	15
1 Introduction	17
1.1 Overview of rotavirus.....	17
1.1.1 Global burden of rotavirus.....	17
1.1.2 Rotavirus structure and classification.....	18
1.1.3 Rotavirus pathogenesis.....	21
1.1.4 Natural immunity to rotavirus	23
1.1.5 Passive immunity to rotavirus infection	28
1.2 Rotavirus vaccines.....	31
1.2.1 Design and development of rotavirus vaccines.....	31
1.2.2 Efficacy of current rotavirus vaccines	32
1.2.3 Correlates of protection for rotavirus vaccines.....	34
1.2.4 Vaccine virus faecal shedding as a measure of vaccine take.....	37
1.2.5 Global introduction of rotavirus vaccines.....	39
1.2.6 Effectiveness and global impact of RV1 and RV5	40
1.2.7 Other rotavirus vaccines	41
1.3 Potential contributory factors to reduced rotavirus vaccine effectiveness	43
1.3.1 Overview of potential contributory factors.....	43
1.3.2 Factors relating to viral epidemiology	44
1.3.3 Factors which reduce overall host immune response.....	46
1.3.4 Factors which reduce specific immune response to rotavirus vaccine	48
1.3.5 Strategies to improve rotavirus vaccine effectiveness in low-income settings	69

1.4	Predicting rotavirus vaccine response in Malawi: study rationale	72
1.5	Overall Hypothesis	74
1.6	References.....	74
2	General Methods.....	94
2.1	Study Context	94
2.1.1	Malawi Demographics and Child Morbidity/Mortality	94
2.1.2	Blantyre demographics and local health care	95
2.2	Study Design	96
2.3	Ethical Approval	97
2.4	Consent	97
2.4.1	General principles of consent	97
2.4.2	Co-recruitment with other studies	98
2.5	Recruitment.....	99
2.5.1	Study Sites.....	99
2.5.2	Cohort study population	100
2.5.3	Case:control study population.....	101
2.6	Social and demographic data collection	107
2.7	Summary of laboratory methods	109
2.8	Specimen Collection	110
2.9	Initial Sample Processing.....	111
2.10	Rotavirus Immunochromatography.....	112
2.11	Enteric Virus Detection: Molecular Laboratory Methods.....	113
2.11.1	Molecular Laboratory Work: Quality Control	113
2.11.2	Nucleic acid extraction.....	114
2.11.3	Reverse Transcription	116
2.11.4	Rotavirus VP6 qRT-PCR	116
2.11.5	Rotavirus Genotyping	117
2.11.6	Rotarix™ vaccine specific (NSP2) RT-PCR.....	122
2.11.7	OPV Multiplex RT-PCR.....	123
2.12	Rotavirus Serology.....	124
2.12.1	Rotavirus-specific IgA ELISA	124

2.12.2	Rotavirus-specific IgG ELISA.....	128
2.13	Poliovirus-specific neutralizing antibody assays	128
2.14	HBGA phenotyping.....	128
2.15	HBGA FUT2 Genotyping	133
2.16	Data Management	136
2.17	Statistical Analysis	136
2.18	Sample size.....	137
2.19	References.....	139
3	Predictors of vaccine virus replication	143
3.1	Introduction.....	143
3.2	Aims.....	145
3.2.1	Hypotheses	145
3.3	Methods	146
3.3.1	Recruitment and data collection	146
3.3.2	Laboratory methods	147
3.3.3	Statistical analysis	148
3.4	Results	149
3.4.1	Recruitment	149
3.4.2	Demographics of shedding cohort.....	150
3.4.3	Rotavirus vaccine virus faecal shedding following RV1 immunisation.....	153
3.4.4	Wild-type rotavirus infection and diarrhoea around time of vaccination	157
3.4.5	Passively-acquired maternal antibody and RV1 vaccine virus shedding.....	158
3.4.6	Oral polio vaccine response and RV1 vaccine virus shedding	159
3.4.7	HBGA phenotype and RV1 vaccine virus shedding.....	166
3.4.8	Demographic and socioeconomic factors	169
3.4.9	Multivariable analysis of predictors of RV1 vaccine virus shedding.....	171
3.5	Discussion.....	172
3.6	References.....	188
4	Predictors of RV1 Immunogenicity.....	193
4.1	Introduction.....	193
4.2	Aims.....	197

4.2.1	Hypotheses	197
4.3	Methods	198
4.3.1	Laboratory methods	199
4.3.2	Statistical analysis	201
4.4	Results	201
4.4.1	Recruitment	201
4.4.2	Demographics of the immunogenicity cohort.....	202
4.4.3	IgA response following RV1 immunisation at 6 and 10 weeks.....	204
4.4.4	RV-specific IgA response and RV1 vaccine virus shedding	204
4.4.5	Passively-acquired maternal antibody and RV-specific IgA response	206
4.4.6	Oral polio vaccine response and RV-specific IgA response	208
4.4.7	HBGA phenotype and RV-specific IgA response	210
4.4.8	Demographic and socio-economic factors	213
4.4.9	Multivariable model.....	215
4.5	Discussion	215
4.6	References.....	227
5	Predictors of Clinical Protection	234
5.1	Introduction.....	234
5.2	Aims	238
5.2.1	Hypotheses	238
5.3	Methods	239
5.3.1	Study design	239
5.3.2	Study Population	239
5.3.3	Demographic, anthropometric and socioeconomic data collection	240
5.3.4	Laboratory methods	241
5.3.5	Statistical Analysis.....	242
5.4	RESULTS.....	244
5.4.1	Recruitment of cases and controls	244
5.4.2	Genotype of rotavirus gastroenteritis cases and asymptomatic RV infection	245
5.4.3	HBGA phenotype and genotype-specific susceptibility to rotavirus GE	247
5.4.4	HBGA phenotype and odds of vaccine failure	251

5.4.5	RV-specific IgA and odds of vaccine failure	254
5.4.6	RV-specific IgA response to natural infection in RV vaccine failures	255
5.4.7	Demographic and socio-economic predictors of RV1 vaccine failure	257
5.4.8	Multivariable model.....	262
5.5	Discussion	263
5.6	References.....	279
6	Conclusions.....	285
6.1	Overview	285
6.2	Summary of Principal findings.....	286
6.2.1	Oral vaccine response in Malawian infants	286
6.2.2	HBGA phenotype	288
6.2.3	Maternal antibody	290
6.2.4	Concurrent OPV	290
6.2.5	Other factors predicting vaccine response.....	292
6.3	Limitations.....	292
6.4	Policy Implications.....	293
6.4.1	HBGA phenotype	293
6.4.2	Maternal antibody	293
6.4.3	Concurrent OPV	295
6.5	Research Implications and future directions	296
6.5.1	Research implications	296
6.5.2	Future directions.....	297
6.6	Conclusion	300
6.7	References.....	301

LIST OF FIGURES

Figure 1-1: Structure of rotavirus triple layer particle.....	19
Figure 1-2: Global distribution of rotavirus genotypes	20
Figure 1-3: Map of countries with rotavirus vaccine introduction.....	40
Figure 1-4: Factors proposed to determine rotavirus vaccine efficacy	44
Figure 1-5: Secretor and Lewis Phenotype Pathway	58
Figure 2-1: Overview of study design	96
Figure 2-2: Flow chart of integrated recruitment of VacSurv and RotaRITE studies.....	98
Figure 2-3: City of Blantyre Community Control Recruitment Map	104
Figure 2-4: Zingwangwa Community Control Recruitment Map.....	105
Figure 2-5: Madziabango Community Control Recruitment Map	106
Figure 2-6: Summary of Cohort Laboratory Methods	109
Figure 2-7: Summary of case control laboratory methods.....	110
Figure 2-8: Illustrative example of HBGA FUT2 genotyping	136
Figure 3-1: Factors which may inhibit rotavirus vaccine virus replication	145
Figure 3-2: Recruitment Flow Diagram for Shedding Cohort	149
Figure 3-3: Peak vaccine virus shedding by shedding pattern	154
Figure 3-4: RV1 vaccine virus shedding patterns.....	155
Figure 3-5: OPV shedding patterns	161
Figure 3-6: Conceptual framework of RV vaccine virus shedding	173
Figure 3-7: Conceptual framework of OPV and RV1 vaccine response.....	180
Figure 4-1: Proposed factors contributing to reduced RV-specific IgA response.....	196
Figure 4-2: Recruitment flow diagram for immunogenicity cohort	202
Figure 4-3: RV-specific IgA response by level of shedding after 1st dose of RV1	205
Figure 4-4: RV-specific IgA response by IgG quartile	207
Figure 4-5: Conceptual framework of OPV and rotavirus vaccine response.....	222
Figure 5-1: Proposed factors contributing to clinical vaccine failure	237
Figure 5-2: Screening and recruitment for case control study.....	244
Figure 5-3: Rotavirus viral load in RV GE cases and asymptomatic RV infection	246
Figure 5-4: Common Genotypes in RV GE cases and asymptomatic RV infection	247
Figure 5-5: Comparison of RV-specific IgA between RV vaccine failures and controls	255
Figure 5-6: Convalescent rise in IgA GMC in RV Cases	256
Figure 6-1: Proposed rotavirus vaccine challenge study	299

LIST OF TABLES

Table 1-1: Other rotavirus vaccines	42
Table 1-2: In-vitro studies of HBGA binding to rotavirus.....	60
Table 1-3: Epidemiological studies of HBGA type and rotavirus	63
Table 1-4: Potential intervention strategies to improve rotavirus vaccine effectiveness	71
Table 2-1: Key social and demographic indicators in urban Malawi	108
Table 2-2: Mastermix for Reverse Transcription	116
Table 2-3: Rotavirus VP6 qRT-PCR Primer and Probe composition.....	117
Table 2-4: Mastermix for Rotavirus VP6 qRT-PCR	117
Table 2-5: Mastermix for G-typing consensus PCR.....	118
Table 2-6: Composition of primers for RV genotyping	119
Table 2-7: Mastermix for G-Typing Multiplex PCR	120
Table 2-8: Mastermix for P-typing consensus PCR	120
Table 2-9: Mastermix for P-Typing Multiplex PCR.....	121
Table 2-10: Composition of NSP2 RT-PCR Primers and Probe.....	122
Table 2-11: Mastermix for NSP2 RT-PCR	122
Table 2-12: Composition of OPV primers and probes	123
Table 2-13: Polio vaccine multiplex RT-PCR mastermix	124
Table 2-14: Preparation of solutions for rotavirus specific IgA ELISA	125
Table 2-15: HBGA phenotyping criteria	130
Table 2-16: Composition of FUT2 primers and probes.....	134
Table 2-17: Mastermix for FUT2 PCR.....	135
Table 2-18: Mastermix for FUT2 restriction step.....	135
Table 2-19: Sample size estimates for Case:Control study.....	138
Table 3-1: Demographics of Shedding Cohort.....	151
Table 3-2: Demographics of cohort infants, compared to those who withdrew or defaulted ..	152
Table 3-3 RV1 shedding: complete data versus incomplete data	153
Table 3-4: Prediction of overall shedding by sample day.....	156
Table 3-5: Relationship between RV-specific IgG and RV1 shedding	158
Table 3-6: Predominant Sabin sub-type shedding pattern.....	160
Table 3-7: Sabin 1 virus shedding and type 1 poliovirus neutralizing antibody response	162
Table 3-8: Sabin 3 virus shedding and type 3 poliovirus neutralizing antibody response	163
Table 3-9: Risk ratio for RV1 shedding by OPV shedding	164
Table 3-10: Correlation between peak shedding of RV1 and Sabin sub-types	164
Table 3-11: Poliovirus-specific neutralizing antibody and RV1 shedding.....	165
Table 3-12: Distribution of secretor and Lewis negative phenotype in shedding cohort*	166
Table 3-13: Relationship between HBGA phenotype and RV1 shedding.....	167
Table 3-14: RV1 vaccine virus shedding by stratified secretor and Lewis phenotype	167
Table 3-15 Median Ct Value for peak shedding by HBGA phenotype.....	168

Table 3-16: RV1 vaccine virus shedding by ABO type.....	168
Table 3-17: Demographic and socio-economic predictors of RV1 vaccine virus shedding.....	170
Table 3-18: Predictors of RV1 vaccine virus shedding: multivariable analysis	171
Table 4-1: Demographic characteristics of immunogenicity cohort	203
Table 4-2: OPV shedding and RV1 seroconversion.....	208
Table 4-3: Poliovirus-specific neutralizing antibody response and RV1 seroconversion	209
Table 4-4 Distribution of secretor/ Lewis negative phenotype in seroconversion cohort	210
Table 4-5: Risk ratio of seroconversion by HBGA phenotype.....	211
Table 4-6 Risk ratio of seroconversion stratified by secretor/Lewis negative phenotype.....	211
Table 4-7: RV1 seroconversion by ABO type	212
Table 4-8: Risk ratio of seroconversion by infant and socioeconomic predictors	214
Table 4-9: Predictors of seroconversion: multivariable analysis	215
Table 5-1: Uncommon rotavirus genotypes and mixed infections	247
Table 5-2: Distribution of secretor/Lewis phenotype in analysis of RV genotype and HBGA....	248
Table 5-3: Odds of genotype-specific RV infection by HBGA phenotype	249
Table 5-4: P[8] rotavirus by secretor/Lewis negative phenotype	250
Table 5-5: P[4] and P[6] rotavirus by secretor/Lewis negative phenotype.....	251
Table 5-6: Distribution of secretor/Lewis negative phenotype in RV GE cases and controls	252
Table 5-7: RV vaccine failure by secretor/Lewis negative phenotype.....	252
Table 5-8: RV Vaccine Failure by ABO phenotype	253
Table 5-9: RV-specific IgA distribution in control groups	254
Table 5-10: Baseline RV-specific IgA in infants with and without convalescent serology	256
Table 5-11: Demographic and socio-economic factors: RV GE cases and community controls	258
Table 5-12: Demographic and socio-economic factors: RVGE cases and non-RV GE controls ..	260
Table 5-13 Demographic and socio-economic factors: all GE cases and community controls ..	261
Table 5-14: Multivariable model of predictors of vaccine failure	262
Table 6-1: Conceptual framework: summary of sub-group characteristics	286
Table 6-2: Advantages and disadvantages of rotavirus delayed dosing regimes.....	294

1 INTRODUCTION

1.1 OVERVIEW OF ROTAVIRUS

1.1.1 Global burden of rotavirus

Rotavirus is the commonest cause of severe diarrhoeal disease in children (Turner et al., 2013, Tate et al., 2012). Rotavirus was first identified in duodenal epithelial cells of children with severe dehydrating diarrhoea by Ruth Bishop and her team at the Royal Hospital for Children, Melbourne in 1973 (Bishop, 2009). Following this discovery, rotavirus was identified as the cause in around 40% of children under five years hospitalized with diarrhoea worldwide (World Health Organization, 2008). In the Global Enteric Multicenter Study (GEMS), the largest study to date determining the burden and aetiology of diarrhoeal disease in sub-Saharan Africa and south Asia, rotavirus was the most common cause of moderate-severe diarrhoeal disease in infancy (Kotloff et al., 2013).

Prior to the 2009 WHO recommendation to introduce universal rotavirus immunisation worldwide, rotavirus was estimated to account for over 450,000 deaths per year in children under five years old. At the time rotavirus resulted in 37% of deaths attributable to diarrhoea, and 5% of all deaths, in young children. Almost all these deaths occurred in low- and middle-income countries in sub-Saharan Africa and south Asia (Tate et al., 2012). The majority of severe rotavirus gastroenteritis occurs in the first year of life, with death primarily due to severe dehydration (Steele et al., 2016).

Rotavirus is not only a major cause of child mortality, but also causes a huge global burden of child morbidity. Parashar et al. (2003) estimated that in 2003, rotavirus caused 111 million episodes of gastroenteritis requiring home care, 25 million episodes requiring a clinic visit and 2 million hospitalisations in children under five years old worldwide each year. Although deaths were rare in higher income settings, the incidence of severe rotavirus diarrhoea was similar in industrialised and developing countries, leading to the conclusion that improvements in hygiene and sanitation alone would be insufficient to reduce the global burden of rotavirus disease. The individual and societal cost impact of rotavirus disease is high. Rheingans et al.

(2009) estimated direct medical costs of \$325 million and total societal costs of \$423 million per year in low and middle-income countries, and predicted that vaccination would be highly cost-effective. Proportionate financial impact may be greater for poorer families and resource-limited health care systems.

Given the huge burden of child morbidity and mortality due to rotavirus, the development and global introduction of safe and effective vaccines should have a major public health impact. However, despite this welcome progress, concerns remain that currently available vaccines are less effective in low-income, high-burden countries than in higher income settings (Soares-Weiser et al., 2010). Limitation of vaccine effectiveness in the very countries which need rotavirus vaccine most could result in a significant continuing burden of rotavirus deaths and disease (see 1.2.2).

1.1.2 Rotavirus structure and classification

Rotavirus is a double-stranded RNA virus comprising an eleven-segment genome surrounded by a non-enveloped triple-layer protein capsid. The genome encodes the six structural proteins (VP1-4, VP6-7) and six non-structural proteins (NSP1-6).

The outer capsid layer is formed by the glycoprotein VP7 shell and protease-sensitive VP4 spike proteins (Figure 1-1). For viral attachment and infection to occur, the VP4 spike protein must be proteolytically cleaved into two sub-fragments, VP5* and VP8*, forming the stalk and head of the spike, respectively (Arias et al., 2015). Although the process of rotavirus entry into cells is not fully understood, both sub-fragments, anchored by the shell glycoprotein VP7, seem to be required for viral attachment and entry to the host cell (Settembre et al., 2011, Mendez et al., 1996). Rotavirus attachment to host cells is primarily mediated through the VP8* domain and binding molecules on the host cell surface. These binding molecules include sialoglycans (such as GM1 and GD1a) and histo-blood group antigens (see 1.3.4.6). During infection, the outer-capsid layer is molecularly transformed and stripped, facilitating entry of the remaining double-layered particle into the cytosol of the host cell. The double-layered particle comprises the intermediate capsid layer structural protein VP6, forming an icosahedral lattice surrounding the core scaffolding protein VP2. The core contains the genome, a viral RNA-dependent RNA

polymerase (VP1) and an mRNA capping enzyme (VP3), allowing viral replication within the host cell (Patton and Spencer, 2000)(Figure 1-1).

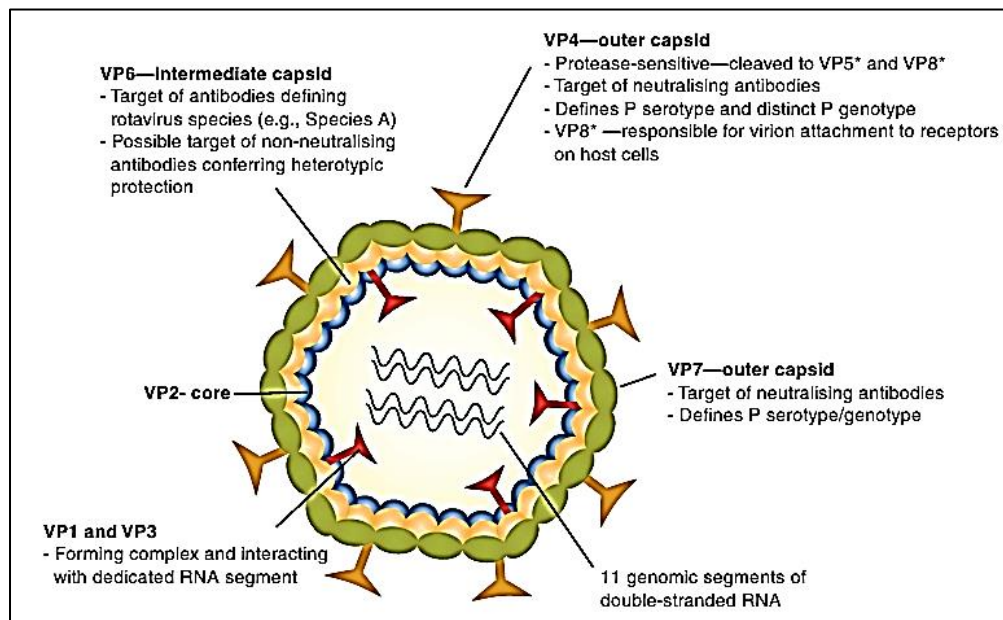


Figure 1-1: Structure of rotavirus triple layer particle

Structure of the rotavirus triple layer particle illustrating the known and potential targets of neutralizing antibodies. VP4 and VP7 are the targets of P-type-specific and G-type-specific homotypic neutralizing antibodies respectively. In addition, conserved epitopes of VP4 and VP7 may determine aspects of heterotypic protection. Anti-VP6 antibodies are produced following infection and may also contribute to heterotypic protection. Figure reproduced with permission from review by Clarke and Desselberger (2015).

Rotaviruses can be classified according to differences in epitopes in the intermediate layer capsid protein VP6 into groups A-G and major sub-groups I and II (Iturriza Gomara et al., 2002). The vast majority of human rotaviruses causing disease are in Group A, sub-group II (Iturriza Gomara et al., 2002).

Rotaviruses can also be categorized using a binary serotype classification system, according to neutralizing antibody response to VP7 and VP4. Rotavirus are described by combinations of G (VP7-specific, G for glycoprotein) and P (VP4-specific, P for protease-sensitive) type. Although initially determined by serology, and termed “serotypes”, equivalent categorization is now more commonly determined by molecular methods, and termed “genotype”. At least 32 G types and 47 P types have been reported, although not all strains infect humans (Crawford et al., 2017). Up to 85 different G and P combinations have been implicated in human

gastroenteritis worldwide, however six common genotypes currently account for around three-quarters of reported human infections: G1P[8]; G2P[4]; G3P[8]; G9P[8]; G4P[8]; and G12P[8] (Doro et al., 2014). Greater genotypic diversity is seen in the African region, with a higher frequency of P[6] genotypes reported (Todd et al., 2010, Doro et al., 2014), (Figure 1-2)

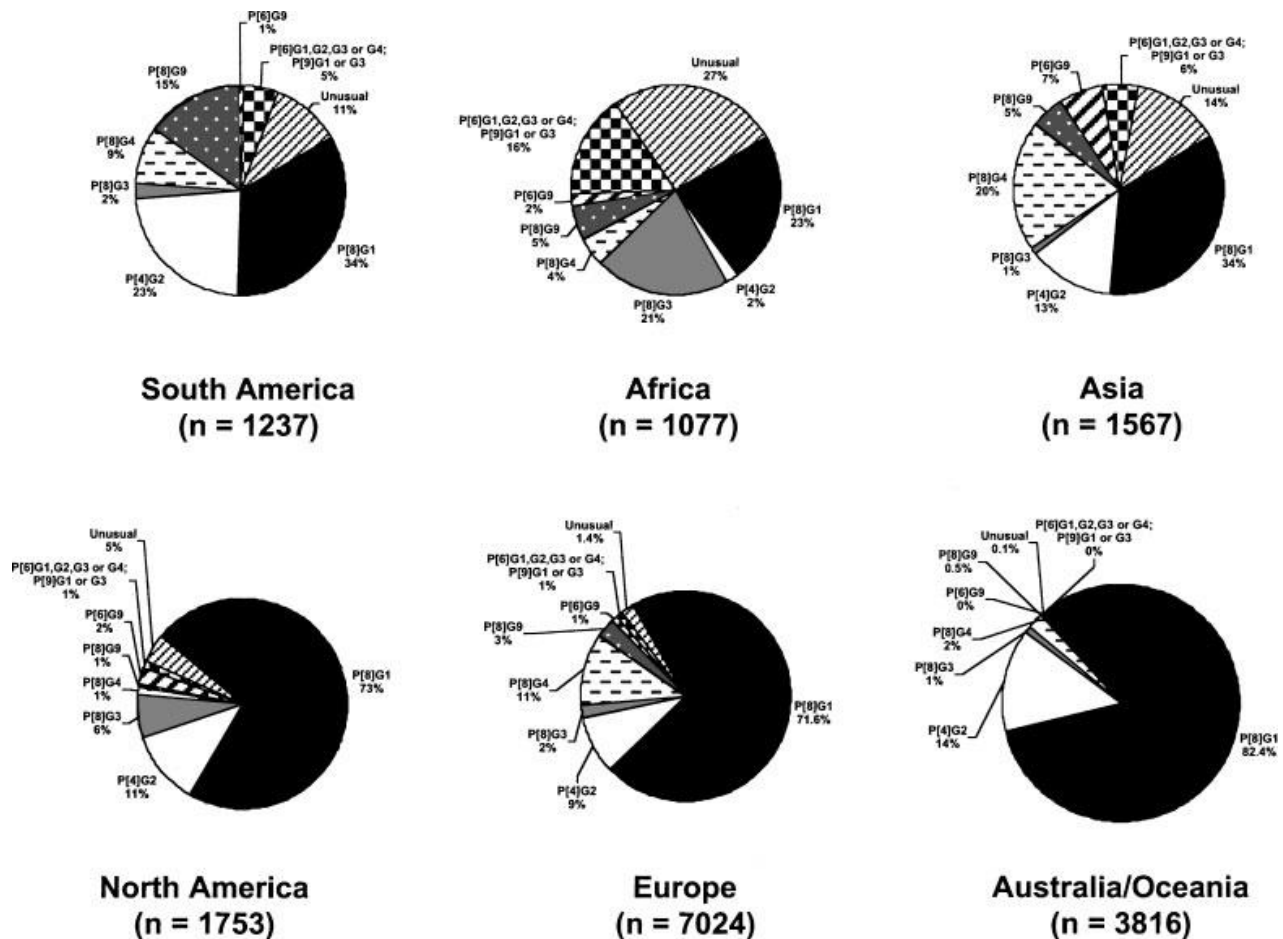


Figure 1-2: Global distribution of rotavirus genotypes

Note the much higher proportion of P[6] and “unusual” rotavirus strains in Africa compared to other regions. Reproduced with permission from Santos and Hoshino. (2005).

The segmented nature of the rotavirus genome allows for reassortment between different human strains, and between human and some animal strains. During co-infection with different but genetically compatible strains, entire genome segments are exchanged. The open reading frame of the gene, and consequently protein integrity, is maintained. Following segment reassortment, the new eleven segment genome is packaged into an intermediate form and

then converted to double-stranded RNA before final morphogenesis into the new virion. The packaging process of new genomes is not well understood, but successful reassortment is thought to rely on highly conserved sequences encoding packing signals within terminal regions of the genes (McDonald et al., 2016). This reassortment plays a major role in the genomic diversity of rotavirus. It has also been exploited in vaccine production, to generate human-animal reassortant strains such as the pentavalent rotavirus vaccine strain, a human-bovine reassortant (see section 1.2.1).

A more complex classification system based on all 11 genomic RNA segments has been proposed and offers opportunities to examine the complexities of viral evolution, but the binary classification remains in common use and informs current understanding of rotavirus immunity and vaccine approaches (Matthijnssens et al., 2008).

1.1.3 Rotavirus pathogenesis

Diarrhoea, defined as three or more loose or liquid stools per day, is the primary clinical feature of rotavirus disease. Vomiting is also a common feature, contributing to the development of severe dehydration which is the primary cause of death and severe morbidity.

Several mechanisms may be associated with rotavirus diarrhoea; osmotic diarrhoea, secretory diarrhoea, altered gut motility and increased gut permeability (Hagbom and Svensson, 2016) .

Enterocytes produce digestive enzymes and absorb solutes and water by both passive diffusion along electrochemical or osmotic gradients and by active transport mechanisms. Disruption of this process leads to malabsorption, with undigested saccharides, carbohydrates, fats and proteins transported to the colon. This high osmotic load unbalances colonic passive transport mechanisms, reducing water absorption and resulting in osmotic diarrhoea.

Rotavirus primarily infects villus enterocytes of the small intestine. Rotavirus infection has been shown to cause enterocyte damage and villous atrophy, which could disrupt the absorptive process, but evidence to confirm a correlation with clinical symptoms is limited by a lack of appropriate models for human infection (Crawford et al., 2017).

In addition to important roles in rotavirus replication and morphogenesis, the enterotoxin NSP4 may have multiple roles in the pathogenesis of rotavirus. Various structures of NSP4, including both intracellular and extracellular forms, have now been identified and studies to determine the functions are ongoing (Sastri et al., 2016). NSP4 may contribute to enterocyte damage via induction of enterocyte apoptosis and disruption of cellular calcium homeostasis during viral replication (Sastri et al., 2016). NSP4 mediated changes in cellular calcium homeostasis also appear to disrupt the cell actin cytoskeleton, leading to loss of cell integrity and polarized epithelial tight cell junctions. This could lead to increased gut permeability (Sastri et al., 2016). Inhibition via NSP4 could also result in reduced activity of sodium-solute co-transporters (Halaihel et al., 2000), although the proven efficacy of oral rehydration solution, a treatment which relies on solute-water co-transport, in the management of rotavirus diarrhoea, casts doubt on the relative contribution and clinical significance of this mechanism in humans (Hagbom and Svensson, 2016). Both enterocyte damage and reduced sodium-solute co-transport could contribute to the osmotic component of rotavirus diarrhoea.

NSP4 may also be important in inducing secretory diarrhoea in rotavirus infection. NSP4 binding to the intestinal epithelium has been shown to induce cell-signalling pathways leading to calcium-dependent chloride secretion in intestinal crypt cells (Sastri et al., 2016). Increased chloride secretion leads to secretory diarrhoea through co-transport mechanisms with water. NSP4 may also contribute to secretory diarrhoea through nitric oxide mediated induction of the enteric nervous system. The enteric nervous system (ENS) controls motility, secretion and blood supply in the intestine, and stimulation triggers secretory reflexes which may contribute to secretory diarrhoea. Enterochromaffin cells, the key sensory cells of the ENS, release 5-hydroxytryptamine (5-HT). Release of 5-HT in rotavirus infection may contribute to secretory diarrhoea and may also trigger vomiting, through interaction with vomiting centres in the central nervous system (Hagbom and Svensson, 2016).

Extra-intestinal clinical complications of rotavirus infection have been reported, most commonly neurological manifestations such as seizures and encephalopathy (Rivero-Calle et al., 2016). Detection of rotavirus antigens and RNA in blood and cerebro-spinal fluid in children with gastroenteritis has been well-documented, although whether this represents systemic

spread of replicating virus is more controversial. Rotavirus viraemia has been detected in 58-72% of children with moderate-severe rotavirus gastroenteritis, and reported rates of antigenaemia are even higher (Rivero-Calle et al., 2016). Potential mechanisms of extra-intestinal spread and pathogenesis are little understood. Post rotavirus vaccine introduction surveillance studies have shown some evidence of a reduction in hospitalization due to seizures in infants, but whether this represents a reduction in primary seizures due to rotavirus or secondary febrile seizures remains unclear (Payne et al., 2014, Pardo-Seco et al., 2015). In either case, it is clear rotavirus infection may cause morbidity beyond diarrhoea. As will be discussed below, it is also clear that rotavirus infection stimulates a systemic as well as mucosal immune response.

1.1.4 Natural immunity to rotavirus

Cohort studies observing the natural history of rotavirus infection have consistently demonstrated that prior infection reduces the risk and severity of subsequent rotavirus disease (Bernstein et al., 1991, Ward and Bernstein, 1994, Velazquez et al., 1996a, Fischer et al., 2002, Gladstone et al., 2011, Mohan et al., 2017). For reasons which are not yet fully understood, naturally acquired immunity confers most protection against subsequent moderate-severe disease, lesser protection against mild disease, and least protection against asymptomatic infection. Direct comparison between different cohort studies is difficult due to methodological issues and small sample size in earlier studies. However, comparison between the three largest and most comparable birth cohort studies suggests the number of infections required to confer protection, and degree of protection attained, may vary between settings. In a cohort study in Mexico, a single prior infection conferred protective efficacy against any rotavirus diarrhoea of 77% (95%CI 60-88%), while a similar study in India found protective efficacy of only 43% (95% CI 24-56%)(Velazquez et al., 1996b, Gladstone et al., 2011). Similarly, in the Mexican cohort two prior infections conferred complete protection against subsequent moderate-severe rotavirus diarrhoea, while in the Indian cohort, two infections conferred only 57% (95%CI 6-80%) and three infections 79%(95%CI 29-94%) protective efficacy. The multi-country MAL-ED cohort study found similar results to the Indian cohort. In unvaccinated infants in Bangladesh, Brazil, India, Nepal, Peru, Pakistan, South Africa and Tanzania, one, two and three prior rotavirus

infections respectively conferred 43%, 62% and 74% protection against subsequent rotavirus infection, although protection against disease was not significant (Mohan et al., 2017). Of note, infants acquired rotavirus infection much earlier in the Indian compared to the Mexican cohort, with 53% of Indian infants infected by 6 months, compared to 34% of Mexican infants. It is possible that earlier acquired rotavirus infections do not stimulate the same host immune response, either due to inhibition by maternal antibody or immaturity of the developing infant immune system. Prevalence of co-infections, environmental enteropathy, malnutrition or genetic factors could also affect the acquisition of natural immunity in different populations. These population differences would later be echoed in vaccine efficacy studies.

Protective mechanisms in natural immunity to rotavirus are still being explored, with mechanistic studies limited by the lack of appropriate or consistent animal models for human infection (Clarke and Desselberger, 2015). Initial virus replication in the intestinal mucosa prompts recognition of viral components by host pattern recognition receptors. This triggers the innate antiviral immune response with increased expression of immunomodulatory proteins and pro-inflammatory cytokines, predominantly Type I and III interferons (Holloway and Coulson, 2013). Host innate immune pathways can directly inhibit steps in the virus replication cycle, and activate and modulate the adaptive immune response through T-cell priming (Liu et al., 2009). Rotaviruses have developed multiple mechanisms, mediated by NSP1, to evade the innate immune response, including induction of degradation of interferon regulatory transcription factors, inhibition of transcription factor NF- κ B, and inhibition of the Jak-STAT signalling pathways (Liu et al., 2009). Rotavirus may also suppress the anti-viral T-cell response, particularly in children (Wang et al., 2007). Although rotavirus specific CD4⁺ T cells expressing mucosal homing receptors have been identified, the quality and magnitude of both CD4⁺ and CD8⁺ T-cell responses appear to be relatively poor (Parra et al., 2014).

The humoral immune response generated against rotavirus infection in humans is mediated by both neutralizing and non-neutralizing antibodies. Antibodies can be homotypic (specific to one rotavirus serotype) or heterotypic (blocking two or more rotavirus serotypes). The relative importance of homotypic and heterotypic antibody responses to rotavirus infection was historically a subject of debate, and shaped different approaches to vaccine development (see

below). There is some evidence to suggest that initial rotavirus infection may generate a predominantly homotypic response, with subsequent infections generating more heterotypic protection (Gorrell and Bishop, 1999, O'Ryan et al., 1994). Although the major protective responses are likely to be mucosal, systemic antibody responses are also generated.

Homotypic rotavirus neutralizing antibodies (RV-NA) target specific epitopes of the outer capsid proteins VP4 and VP7 (Figure 1-1). The presence of these neutralizing antibodies within the intestine is thought to confer strain-specific protection against rotavirus infection (Angel et al., 2014), however measurement of intestinal neutralizing antibodies is technically challenging. Studies determining the correlation between serum homotypic and heterotypic RV-NA and protection against natural infection have shown variable results. One cohort study in an orphanage showed a correlation between homotypic RV-NA and protection against subsequent rotavirus gastroenteritis (Chiba et al., 1986). Similarly, another cohort study in a day care centre found a protective effect against rotavirus infection (O'Ryan et al., 1994). However, a large case:control study in Bangladesh found that despite evidence of lower homotypic and heterotypic RV-NA response in rotavirus cases, only heterotypic antibodies were significantly associated with protection against rotavirus disease (Ward et al., 1992). In a recent functional analysis of antibodies cloned from human intestinal rotavirus-specific B cells, Nair et al. (2017) identified antibodies to the VP5* stalk fragment of VP4 as potential mediators of heterotypic immunity. Heterotypic antibodies to VP7 and the VP8* fragment of VP4 were also identified, but were less commonly expressed than homotypic anti-VP7 and non-neutralizing anti-VP8* responses. Improved understanding of targets of heterotypic protection could aid future vaccine development.

Non-neutralizing rotavirus-specific IgA and IgG antibodies also form an important part of the rotavirus immune response. Non-neutralizing antibodies against the conserved intermediate capsid protein VP6 (Figure 1-1), the most abundant antibodies produced by rotavirus-specific B cells in infants following natural infection, are thought to play a major role in heterotypic immunity (Svensson et al., 1987, Aiyegbo et al., 2013, Corthesy et al., 2006). Initial murine models suggested that non-neutralizing anti-VP6 secretory IgA could bind rotavirus

intracellularly resulting in viral expulsion from the enterocyte (Burns et al., 1996) and could inhibit viral transcription (Feng et al., 2002). A more recent experimental model of human infection found similar evidence of intracellular inhibition of rotavirus replication using human anti-VP6 IgA antibodies, and identified a possible binding site (Aiyegbo et al., 2013). In contrast to some other viruses, there is no evidence to date that non-neutralizing antibodies to rotavirus are associated with interference with effective immunity or immunopathology (Rouse and Sehrawat, 2010).

Clarke and Desselberger (2015) summarized proposed mechanisms of the IgA response to rotavirus infection. Plasma cells in the submucosa generate both monomeric rotavirus-specific IgA and dimeric IgA. Both monomeric and dimeric IgA circulate in blood, but dimeric IgA also binds with poly-immunoglobulin receptors (pIgR) on enterocytes of the mucosal epithelium, facilitating transcytosis of IgA into the cell then secretion onto the luminal surface. Proteolysis then allows release of dimeric IgA, still bound to the secretory component of the pIgR, into the intestinal lumen as secretory IgA (sIgA). Within the intestinal lumen, sIgA binds rotavirus, excluding viral entry into the enterocyte and thereby limiting infection. Secretory IgA can be detectable in faeces (copro-IgA). Secretory IgA may also cross back across the mucosal epithelium to re-enter the circulation, allowing detection in blood. Levels of both rotavirus-specific copro-IgA and total rotavirus-specific serum IgA are highly correlated with levels in duodenal secretions and can therefore be used as a proxy for response at the primary site of infection (Grimwood et al., 1988).

There are limited data on the correlation between copro-IgA and protection against rotavirus infection. Matson et al. (1993) found higher levels of rotavirus-specific copro-IgA were associated with protection against both symptomatic and asymptomatic rotavirus infection in children in a day centre. In a small cohort study, Coulson et al. (1992) found that re-infection with rotavirus was associated with the persistence of an elevated “plateau” of copro-IgA in some children, which appeared protective against subsequent disease. In both studies, the correlation was not complete: some children with higher titres became symptomatic and some children with low titres remained well. Similarly, an experimental rotavirus challenge study in adults found no correlation between rotavirus-specific copro-IgA, or jejunal IgA, and protection

against rotavirus infection or disease (Ward et al., 1989). Some of these inconsistencies may be due to technical difficulties in measurement and the rapid degradation of copro-IgA.

In the Mexican birth cohort study of natural rotavirus infection discussed above, serum rotavirus-specific IgA titres above 1:800 were associated with decreased risk of rotavirus infection (adjusted relative risk 0.21) and were associated with complete protection against moderate-severe rotavirus gastroenteritis. These “protective” titres were generally achieved following two symptomatic or asymptomatic infections (Velazquez et al., 2000). A similar association between higher rotavirus-specific serum IgA and protection against infection was observed in a smaller US day centre cohort (O’Ryan et al., 1994).

In the Mexican birth cohort, higher rotavirus-specific IgG (titre >1:6400) was also correlated with protection against infection. The association was not as strong (adjusted relative risk 0.51) as that observed with rotavirus-specific IgA and there was no evidence of protection against rotavirus gastroenteritis. An association between rotavirus-specific IgG and protection against rotavirus gastroenteritis was observed in the smaller US day centre cohort (at titres >1:800) and Bangladesh case:control (at titres of 100-199 U/mL) studies (O’Ryan et al., 1994, Ward et al., 1992). The mechanism of protection by rotavirus-specific IgG is unclear, but transcytosis of IgG into the intestinal lumen could prevent rotavirus attachment or block replication within the intestinal epithelium in a similar manner to secretory-IgA (Westerman et al., 2005).

In understanding the adaptive immune response to rotavirus infection there is growing interest in rotavirus-specific B cell responses. In murine studies, IgM memory B cells are associated with rapid IgM and IgG response following rotavirus infection (Narvaez et al., 2010), however responses in mice frequently differ from those in humans and further research is needed. Rotavirus-specific isotype-switched memory B cells with mucosal homing phenotype have been investigated as correlates of protection in human infection (Rojas et al., 2007). To date, evidence for a role in human protective immunity is limited but this is a promising area of research.

Mechanisms of protective immunity stimulated by natural infection may differ from vaccine-generated immunity. Correlates of protection following rotavirus immunisation are discussed in detail below.

1.1.5 Passive immunity to rotavirus infection

Passive protection through trans-placentally acquired maternal antibody

The age of first rotavirus disease is a function of both host immunity and force of infection. Rotavirus infections in early life are generally asymptomatic, with the first peak of symptomatic infection around 5 months of age in unvaccinated African infants, and 9-10 months in European infants (Steele et al., 2016). The observation that symptomatic rotavirus is uncommon in early infancy, even in settings with a high force of infection and early exposure, strongly supports the existence of passively acquired protective immunity.

Maternal antibodies, acquired through transplacental transfer, provide passive immunity to newborn infants against many infectious diseases in the first few months of life. Transplacental transfer is limited to IgG, and occurs primarily in the 3rd trimester. Trans-placentally acquired maternal antibodies decline exponentially from birth with a half-life of around 30-50 days (Sarvas et al., 1993), and are generally absent by 6-12 months of age.

In a birth cohort study examining the dynamics of rotavirus-specific antibodies from birth to two years of age, Zheng et al. (1989) determined that infants in Hong Kong had a broad spectrum of neutralizing antibodies at birth, which declined to a nadir at 4 months, then rose from 8 months onwards in response to exposure, reaching levels equivalent to those at birth around 12-16 months of age. The nadir in passively-acquired antibody coincided with the peak of symptomatic infection in this population at 5 months old. Ray et al. (2007) in a small birth cohort study determined that maternal rotavirus-specific IgG was lower in infants who developed rotavirus infection in the first 6 months of life compared to infants who did not. In contrast, studies of neonatal rotavirus infections in India (Jayashree et al., 1988b) and South Africa (Haffejee et al., 1990) also found evidence of rotavirus-specific antibodies in neonates, but no evidence that this prevented neonatal rotavirus infections. However, most neonatal infections were asymptomatic, and numbers of infants with gastroenteritis were too small to

determine whether the presence of passively acquired rotavirus-specific antibodies prevented disease. Ramachandran et al. (1998) found evidence of serotype-specific passive immunity - infants who developed the neonatal rotavirus infection G9P[11] had lower cord-blood homotypic neutralizing antibodies to that strain than infants who did not acquire infection. G9P[11] is a common strain in neonates, but rare in the wider community. The authors speculated that reduced maternal exposure to this strain resulted in reduced passively-acquired protection in neonates and might explain the higher neonatal prevalence of this strain. Of note, asymptomatic G9P[11] infection in neonates generated a robust infant immune response, leading to the identification of this strain as a vaccine candidate (see below).

The mechanism of protection by passively acquired rotavirus-specific IgG is unclear, but could be mediated systemically, or through blocking or inhibitory effects on virus replication in the mucosa and intestinal lumen as discussed above (Westerman et al., 2005).

Passive protection through breast-feeding

The World Health Organization recommends exclusive breast-feeding for the first 6 months of life as the optimal way of feeding infants, followed by complementary foods with continued breastfeeding up to two years of age. It has been well established that exclusive breastfeeding reduces the risk of diarrhoeal disease, although the extent to which this protective effect is mediated by immune or anti-microbial components of breast-milk, by effects on the infant microbiome, or by avoidance of unsafe alternative feeds is still subject to debate (Horta and Victora, 2013). Most studies of breast-feeding effects are observational, due to ethical constraints, and are subject to multiple socio-economic confounders.

Maternal secretory IgA in breast milk is thought to act within the intestinal lumen in the same way as the infant's own secretory IgA, by binding pathogens and preventing attachment to enterocytes. The effect is transient and local to the gut. While there is some evidence of intestinal uptake of colostral IgA by preterm infants, reflected in a rise in pathogen-specific serum IgA, there is no evidence that maternal secretory IgA impacts systemic immunity beyond the first days of life (Brandtzaeg, 2003). Cellular components of breast milk, particularly macrophages, may also contribute by release of immunoregulatory cytokines, stimulating the

infants own mucosal response (Slade and Schwartz, 1987). In addition, innate immune factors such as lysozyme, lactoferrin and human milk glycans are increasing thought to be important (Morrow et al., 2005).

Several studies have sought to determine whether breast-feeding reduces the risk of rotavirus infection with variable results. A recent meta-analysis found evidence overall that exclusive breastfeeding (compared to mixed or exclusive formula feeding) did reduce the risk of rotavirus infection in the first 2 years of life (pooled odds ratio 0.62 (95%CI 0.48-0.81). However, the largest case:control study, conducted in Bangladesh, found that exclusive breastfeeding in early life was associated with reduced risk of severe rotavirus diarrhoea in the first year, but a higher risk in the second year of life (Clemens et al., 1993), with no overall protective effect. The authors reflected that severe rotavirus gastroenteritis might be postponed, rather than prevented, by breastfeeding.

The protective mechanisms of breast milk components against rotavirus infection have been explored. In an early Australian study, McLean and Holmes (1981) noted that higher levels of both rotavirus-specific secretory IgA and trypsin inhibitors in maternal breastmilk were associated with reduced risk of rotavirus infection in the first week of life. Jayashree et al. (1988a) confirmed the same results in a small Indian cohort. However, it was noted that IgA and other non-antibody anti-microbial components in breastmilk are found at much higher levels in colostrum, and protective levels might not be maintained in later lactation.

In a cohort study of Mexican infants up to two years of age, Newburg et al. (1998) found that higher levels of the glycoprotein lactadherin prior to infection were associated with asymptomatic compared to symptomatic rotavirus.

Later studies of the effect of breast milk on rotavirus infectivity have focused primarily on vaccine strains. These will be discussed in detail below.

In summary, it is likely that breastfeeding does contribute to protection against rotavirus infection, but that protection is incomplete, and primarily limited to the period of exclusive breastfeeding.

1.2 ROTAVIRUS VACCINES

1.2.1 Design and development of rotavirus vaccines

Live, oral rotavirus vaccines are based on the principles of natural immunity discussed above: that prior infection reduces risk of subsequent severe disease, and that there is some degree of heterotypic protection between serotypes.

Rhesus reassortant tetravalent rotavirus vaccine (RRV-TV)

Initial rotavirus vaccine candidates explored in the 1980's were monovalent animal strains, including rhesus rotavirus or the bovine rotavirus strains RIT 4237 or WC3. These proved to be safe and immunogenic but poorly efficacious, with limited heterotypic protection. This led in the early 1990's to the development of multivalent human-animal rotavirus reassortant vaccine candidates, combining human VP7 or VP4 genes with a bovine or rhesus monkey rotavirus strain backbone (Midthun and Kapikian, 1996). The most successful of these candidates was the rhesus reassortant tetravalent rotavirus vaccine, RRV-TV. This vaccine was comprised of a live attenuated rhesus rotavirus G3 strain, and three reassortant rotaviruses comprising 10 rhesus rotavirus genes and one human VP7 gene determining G1, G2, and G4 serotype specificity. Following promising pre-licensure trials RRV-TV became the first licensed rotavirus vaccine, produced commercially by Wyeth and approved for routine use in the USA in 1998 as "RotaShield®". However, post-licensure adverse events surveillance identified an excess risk of intussusception, and the vaccine was withdrawn the following year (Kapikian, 2001). Despite some discussion about whether the increased risk of intussusception would be outweighed by benefits of reducing rotavirus mortality in high-burden, low-income settings, further trials of RRV-TV became ethically problematic, and new vaccine candidates were sought.

More recently, there has been resurgent interest in RRV-TV in low-income settings with neonatal dosing to reduce the risk of intussusception (Armah et al., 2013). However, more widespread use is unlikely given the growing number of alternatives.

Current internationally licensed rotavirus vaccines (RV1 and RV5)

Two major vaccine candidates emerged in the early 2000's with contrasting designs reflecting debate at that time regarding the extent of heterotypic protection generated by a single rotavirus strain (see 1.1.4).

Based on the sub-optimal heterotypic protection of earlier monovalent animal rotavirus vaccines, the pharmaceutical company Merck continued the multivalent reassortant vaccine approach. This led to the development of a pentavalent vaccine (RV5) comprising five live reassortant rotavirus strains based on a bovine WC3 strain backbone, four expressing the human VP7 types G1, G2, G3 and G4, and a fifth expressing the human VP4 type P[8]. This vaccine was licensed as RotaTeq® and approved by the WHO for routine use in 2008. RV5 is recommended by the manufacturer to be given as a three-dose schedule at 2, 4 and 6 months.

Based on the principle that natural rotavirus infection with a single strain does generate heterotypic protection, the pharmaceutical company GlaxoSmithKline developed a monovalent rotavirus vaccine (RV1) based on an attenuated human G1P[8] strain (RIX4414). This vaccine was licensed as Rotarix® and approved by the WHO for routine use in 2008. RV1 is recommended by the manufacturer to be given as two-dose schedule with first dose after 6 weeks and final dose before 24 weeks of age with at least 4 weeks between doses.

1.2.2 Efficacy of current rotavirus vaccines

In randomized controlled trials, RV1 and RV5 showed similar efficacy. Both vaccines proved highly efficacious in high-income countries, but later trials in lower-income settings identified a significant variation in efficacy between different settings. Efficacy ranged from over 90% in North America and Europe, to around 80-90% in low mortality Asian and North African countries, around 70-80% in Latin American countries and less than 50% in Africa and high-mortality Asian countries (Fischer Walker and Black, 2011). There was clear gradient in efficacy from high efficacy in high-income, low mortality countries, to low efficacy in low-income, high-mortality countries.

A systematic review and meta-analysis in 2012 summarized all randomized trials of RV1 and RV5 up to that year (Soares-Weiser et al., 2012a): a total of 41 trials enrolling a total of 186,263 participants. In view of the wide efficacy gap between high and low-income settings, Soares-

Weiser et al. (2012b) stratified their meta-analysis by low mortality (WHO strata A or B with very low/low child mortality) and high mortality (WHO strata D or E with high child mortality). No studies were undertaken in WHO stratum C countries.

RV1

Twenty-nine trials of RV1 which enrolled a total of 101,671 participants were included in the review and meta-analysis (Soares-Weiser et al., 2012b). These included 19 safety or immunogenicity studies, 11 with short-term efficacy data up to 2 months post vaccine, and 10 studies with primary efficacy outcome measures. Efficacy outcomes included rotavirus diarrhoea of any severity (18 trials), severe rotavirus diarrhoea (11 trials) and rotavirus diarrhoea requiring hospitalization (9 trials). Ten trials included all cause diarrhoea as an outcome measure. Studies were conducted in North America (4 trials), Europe (6 trials), Asia (11 trials), Latin America (6 trials) and Africa (4 trials in two countries, South Africa and Malawi).

Eleven trials provided data on efficacy of RV1 against severe rotavirus diarrhoea, including two studies in high mortality countries (Madhi et al., 2010, Steele et al., 2010b). In low mortality countries pooled vaccine efficacy was 86% at one-year follow-up (95%CI 74-93%, 40631 participants, six trials) and similar after two years (85%, 95%CI 80-88%, 32,854 participants, eight trials). In high mortality countries pooled vaccine efficacy was 63% at one-year follow-up (95%CI 25-82%, 5414 participants, two trials) but only 42% by two years (95%CI 21-58%, 2764 participants, one trial). Of note, Madhi et al. (2010) included data from both South Africa and Malawi. Both are high mortality countries, but South Africa is an upper middle-income country, while Malawi is a low-income country. RV1 efficacy against severe rotavirus gastroenteritis in the first year of life was higher in South Africa (76.9% (95%CI 56.0-88.4%) than in Malawi (49.4% (95%CI 19.2-68.3%).

RV1 efficacy was lower against rotavirus diarrhoea of any severity and against all-cause diarrhoea than against severe rotavirus gastroenteritis in both high and low mortality settings. Similar efficacy gaps to those observed for severe rotavirus gastroenteritis were seen between high and low mortality countries for “any rotavirus diarrhoea” and all-cause diarrhoea outcomes. RV1 was safe with no excess of adverse events or deaths in vaccinated children.

RV5

Twelve trials of RV5 which enrolled a total of 84,592 participants were included in the review and meta-analysis (Soares-Weiser et al., 2012b). These included 4 studies reporting safety or immunogenicity outcomes only, and 8 studies with primary efficacy outcome measures (including 7 with reported immunogenicity outcomes). Efficacy outcomes included rotavirus diarrhoea of any severity (8 trials), severe rotavirus diarrhoea (8 trials) and rotavirus diarrhoea requiring hospitalization (1 trial). Three trials included all cause diarrhoea as an outcome measure. Studies included sites in North America (5 trials), Europe (4 trials), Asia (5 trials), Latin America (1 trial in three countries Guatemala, Mexico, Puerto Rico) and Africa (1 trial in three countries, Ghana, Kenya and Mali).

Six trials provided data on efficacy of RV5 against severe rotavirus diarrhoea, including two studies with stratified data from high mortality countries (Bangladesh, Zaman et al. (2010) and Ghana, Mali and Kenya Armah et al. (2010)). In low mortality countries pooled vaccine efficacy was 87% at one-year follow-up (95%CI 55-96%, 2344 participants, three trials) and 82% after two years (95%CI 50-93%, 3190 participants, three trials). In high mortality countries pooled vaccine efficacy was 57% at one-year follow-up (95%CI 38-71%, 5916 participants, two trials) but only 41% by two years (95%CI 18-57%, 5885 participants, two trials).

RV5 efficacy was lower against rotavirus diarrhoea of any severity or against all cause diarrhoea than against severe rotavirus gastroenteritis in both high and low mortality settings. In high mortality countries (1059 participants, one trial) there was no difference between RV5 and placebo against all cause diarrhoea of any severity. Similar efficacy gaps to those observed for severe rotavirus gastroenteritis were seen between high and low mortality countries for “any rotavirus” and all-cause diarrhoea outcomes. RV5 was safe with no excess of adverse events or deaths in vaccinated children.

1.2.3 Correlates of protection for rotavirus vaccines

While clinical endpoints are the most important measure of any vaccine, large-scale clinical efficacy trials are expensive and logistically challenging. In addition, since current vaccines have proven efficacy, testing newer vaccine candidates against placebo becomes ethically

challenging. Since differences in efficacy between new and old vaccines may be smaller than differences between new vaccine and placebo, even larger sample sizes will be required. Surrogate end-points, biomarkers which substitute for a clinical end-point, may offer a logistically less challenging approach. For rotavirus vaccines, efforts to identify suitable surrogate end-points have primarily focused on determining correlates of protection: immune markers statistically correlated with clinical vaccine efficacy. Correlates of protection may be mechanistic, causally responsible for observed protection, or non-mechanistic, associated with but not directly causally related to protection. A non-mechanistic correlate of protection should still have a plausible biological link to the protective mechanism -for example a serum biomarker that directly correlates with a mucosal mechanism of protection. Correlates of protection may also be absolute, where a given response guarantees protection, or relative, where a given response is generally associated with protection (Angel et al., 2014).

Based on data on natural immunity to rotavirus (see 1.1.4) serum rotavirus neutralizing antibody and serum rotavirus-specific IgA have been investigated as possible correlates of protection following rotavirus immunisation.

Serum rotavirus neutralizing antibodies were poorly correlated with clinical protection in three large trials, underestimating protection provided by RV5 vaccine (Armah et al., 2012, Armah et al., 2010, Vesikari et al., 2006). Similarly, in early trials of 89-12 vaccine, a precursor to RV1, rotavirus neutralizing antibody seroconversion rates were low (Bernstein et al., 1999). This poor correlation is partly due to difficulties in interpretation due to the indistinguishability of passively acquired maternal neutralizing antibody and antibodies produced in response to vaccine. A decline in maternal antibody over time could lead to lower total neutralizing antibody post immunisation, and be incorrectly interpreted as a failure of vaccine response. Laboratory methods cannot distinguish between maternal and actively-acquired infant IgG. Analytic approaches to determine the relative contribution would be complex and logistically challenging in a cohort, and impossible in a cross-sectional case-control design. Serum rotavirus neutralizing antibodies have therefore been largely rejected as reliable correlates of protection for rotavirus vaccines, although are still reported in some studies as a reflection of heterotypic immune response.

Serum rotavirus-specific IgA is better correlated with protection. Measured by sandwich ELISA (see 2.12.1), response is generally reported as either seropositivity or seroconversion.

Seropositivity is defined as >20U/mL, a value chosen in early studies as the cut-off point as it was over five times the lower limit of detection (Bernstein et al., 1999). In studies which use seropositivity post-immunisation as a measure of vaccine response, seroconversion is defined as the appearance of antibodies (>20U/mL) in infants seronegative pre-immunisation. Baseline seropositivity in some high-burden settings is high. In other studies, seroconversion is variably reported as either a three or four-fold rise in titres post immunisation. Timing of post-vaccination sampling for IgA seroconversion varies between studies from 2-8 weeks following the last vaccine dose (Angel et al., 2014).

There are few studies directly assessing serum rotavirus-specific IgA as a correlate of protection. Most were longitudinal cohort sub-studies of larger vaccine trials, limited by relatively small numbers. There was some evidence of correlation between serum rotavirus-specific IgA response and clinical protection against rotavirus infection, but not gastroenteritis, following low-dose RRV-TV vaccine immunisation (Ward et al., 1997). An early RV1 study in Finland found a significant association between IgA seroconversion and protection against rotavirus gastroenteritis (Vesikari et al., 2004b). Cheuvart et al. (2014) used trial data from the Malawi/South Africa RV1 vaccine trial to attempt to validate seroconversion based on seropositivity >20U/mL as a correlate of protection. They found that while seropositivity post-vaccination was associated with protection against rotavirus gastroenteritis, seronegative vaccinated subjects were also protected. IgA seropositivity failed to capture a large proportion of the vaccine effect, suggesting it may be a non-mechanistic correlate of protection. In a second analysis, Cheuvart et al. (2014), performed a meta-analysis of 8 RV1 efficacy trials in varied settings, and found a linear correlation between rotavirus-specific IgA seroconversion and clinical vaccine efficacy. The authors reflected that rotavirus-specific IgA responses might be more reliable as a predictor of population level efficacy than as a measure of individual protection.

Further exploring the relationship between rotavirus-specific IgA and clinical protection at a population level, Patel et al. (2013), performed a meta-analysis of a wide range of RV1 and RV5

immunogenicity studies. There was significant correlation ($r^2=0.56$, $p=0.005$) between rotavirus-specific IgA geometric mean titre (GMT) post-immunisation and vaccine efficacy. Post-immunisation IgA responses followed a similar inverse gradient to vaccine efficacy between high and low-mortality settings, where IgA responses were lowest in high-mortality, and highest in low-mortality countries. Patel et al. (2013) determined a cut-off level of serum rotavirus-specific IgA GMT of $>90\text{U/mL}$ post-immunisation which predicted sub-optimal vaccine performance at a population level. Pooled vaccine efficacy over two years in countries with a median IgA GMT $>90\text{U/mL}$ was 85% (80-90%), compared to 44% (30-55%) in countries with a median IgA GMT $<90\text{U/mL}$. However, in a further review of the same data, Angel et al. (2014) cautioned that there were some differences in the association between IgA responses and clinical protection for RV1 and RV5 vaccines. In RV1 studies, IgA seroconversion rates consistently underestimated clinical protection. However, results were less consistent for RV5 studies, where in some studies IgA seroconversion over-estimated clinical protection. These inconsistencies could represent technical assay differences, different definitions of seroconversion, or different immune responses to the two vaccines.

Alternative correlates of protection for rotavirus vaccines are being explored, including B and T cell responses and VP7 or VP4 specific antibodies (Angel et al., 2014). In the meantime, serum rotavirus-specific IgA remains the best available correlate of protection, although with some limitations.

1.2.4 Vaccine virus faecal shedding as a measure of vaccine take

The presumed mechanism of action of live oral rotavirus vaccines is that vaccine virus replication within the gut stimulates a host immune response. Vaccine virus shedding in stool is considered a pragmatic proxy measure of intestinal vaccine virus replication, and has therefore been reported in some studies as a measure of vaccine take. Vaccine take has been variably defined, but broadly refers to a measure of active vaccine virus replication following immunisation associated with an effective immune response. The principle that vaccine virus shedding correlates well with immune response and clinical protection has been well established for oral polio vaccines (Giri et al., 2018).

A larger number of studies have reported vaccine virus shedding to determine the risk of horizontal transmission of rotavirus vaccine to contacts. However, despite the relative ease and acceptability of collection of stool compared to blood, vaccine virus faecal shedding has been little explored as a surrogate end-point for protection.

In most published studies, vaccine virus faecal shedding has been determined by rotavirus antigen detection by enzyme immuno-assay (EIA), although a few studies have used molecular methods of detection or for confirmation of EIA positive samples. EIA is considerably less sensitive than molecular methods of detection: one study reported detection rates of 80-90% using real-time polymerase chain reaction (RT-PCR), and only 20-30% using EIA (Hsieh et al., 2014).

In a systematic review of studies mostly using EIA for detection of shedding (Anderson, 2008), shedding rates for RV1 were generally higher (around 50%, range 33-80%) than those for RV5 (around 10%, range 3-13%).

The human-bovine reassortant vaccine RV5 is known to replicate less well in the human intestine, but is given at a higher dose to compensate for this. Differences in timing of stool sampling may also have affected RV5 detection rates: the majority of RV5 studies sampled on day 3, whereas RV1 studies tended to sample on day 7. A much higher rate of detection (21.4%) was found in an American study which sampled stool daily for 9 days following RV5 immunisation (Yen et al., 2011).

Of note, vaccine virus shedding rates were generally much higher following the first dose of immunisation than following subsequent doses for both RV1 and RV5. Vesikari et al. (2004a) in Finland reported shedding rates of 52% following the 1st dose of RV1, and only 7% following the 2nd dose, while Phua et al. (2005) in Singapore reported shedding of 76-80% after 1st dose of RV1, compared to 18-29% following the 2nd dose. Similarly, two studies in Latin America reported shedding rates of 35-61% following the 1st dose and 11-21% following the 2nd dose (Salinas et al., 2005, Ruiz-Palacios et al., 2007). Several of these studies also reported that shedding following the 2nd dose was primarily seen in infants who failed to demonstrate an

adequate immune response to the 1st dose. This supports the concept that vaccine virus shedding may be a useful correlate of vaccine take.

Also of interest, shedding rates were lower in post-licensure studies in low-income settings with poorer vaccine efficacy: shedding rates in Bangladesh were only 25% and in Ghana only 5% by EIA (Zaman et al., 2009, Armah et al., 2016). However, EIA may not detect low-level rotavirus shedding. In urban Vellore, RT-PCR detected low-level vaccine virus shedding in 24% of infants post RV1 immunisation (Lazarus et al., 2017).

To date, there are no published data directly assessing vaccine virus faecal shedding as a surrogate end-point for clinical protection.

1.2.5 Global introduction of rotavirus vaccines

In 2009, the WHO recommended that rotavirus vaccines should be included in all national immunisation programmes, particularly in countries with high rotavirus mortality rates, as part of a wider strategy to reduce the burden of diarrhoeal disease (World Health Organization, 2009). WHO made an equal recommendation of either RV1 or RV5, and further recommended that scheduling coincide with existing national immunisation schedules for logistic reasons. For most low-income countries following the extended programme of immunisation (EPI) schedule, this dictates a schedule of immunisation for RV1 at 6 and 10 weeks. RV5 schedules are more variable. Due to concerns relating to intussusception risk in older infants, it was initially recommended that the first dose of rotavirus vaccine should not be given after 15 weeks, and the final dose not given after 32 weeks. However, following a review of safety and effectiveness data, and a reassessment of the balance of risk of mortality from intussusception versus rotavirus, WHO revised this recommendation in 2013 to remove the upper age limit. The 2013 revised recommendation stated that rotavirus vaccines should not be given after 24 months of age, due to the typical age distribution of rotavirus (World Health Organization, 2013).

By March 2017, 85 countries had introduced rotavirus vaccines nationally, 2 were in the process of phased introduction, and 5 had introduced sub-national programmes (Figure 1-3). This included 39 low-income countries with Global Vaccine Alliance (Gavi) support. Progress has been particularly good in Africa, with 64% of countries in this region now providing rotavirus

vaccines, although several high-burden countries including Chad, the Democratic Republic of Congo, Niger and Nigeria have not yet introduced national programmes. Progress has been poor in south-east Asia, where only 18% of countries have introduced national programmes (Rota Council, 2017). Primarily due to the lower cost and convenience of a two-dose schedule, most countries to date have chosen to introduce RV1 rather than RV5 (Burnett et al., 2016).

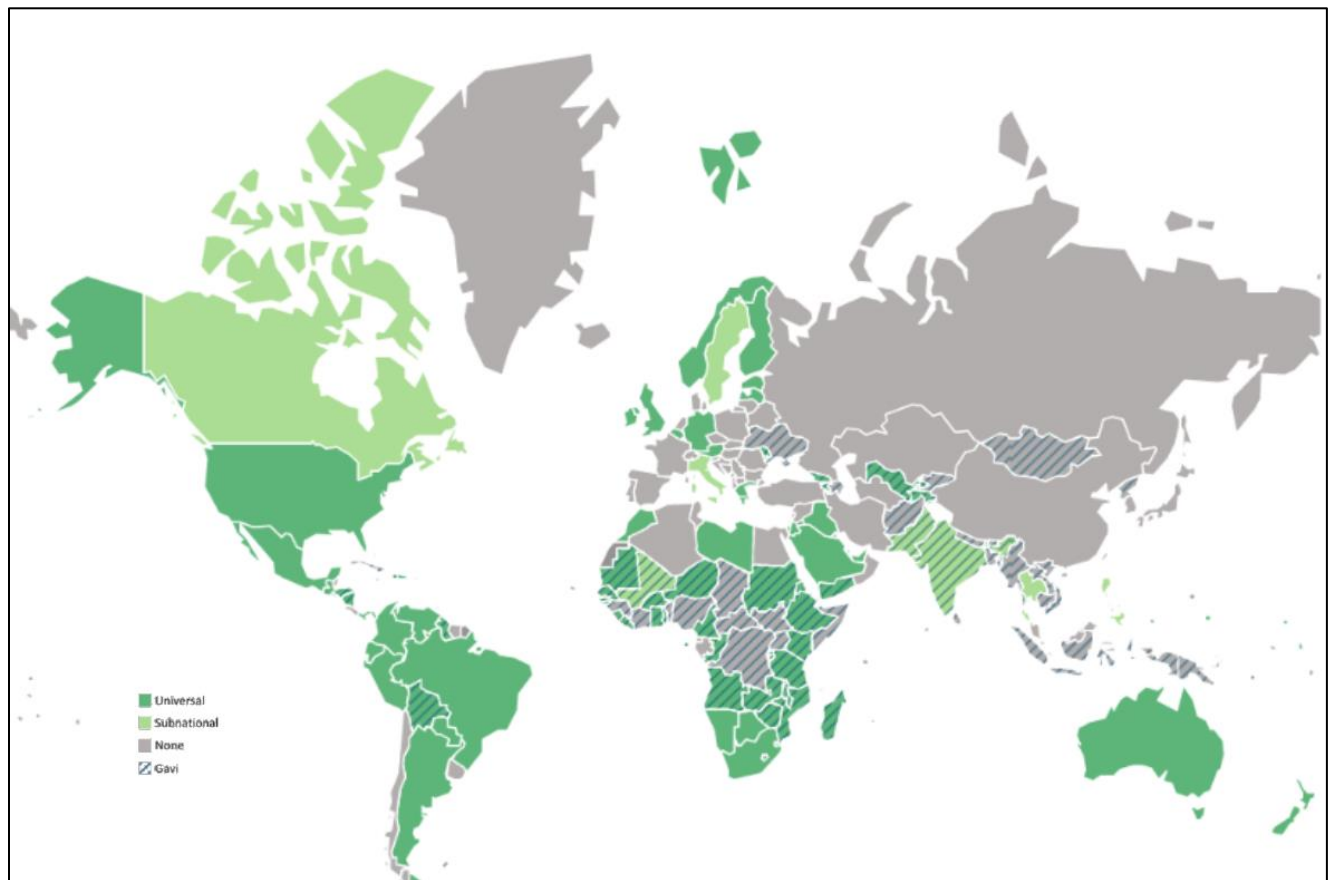


Figure 1-3: Map of countries with rotavirus vaccine introduction

Countries with universal or sub-national introduction of rotavirus immunisation as of March 2017.

Adapted from ROTA Council advocacy resources. <http://rotacouncil.org/resources/advocacy-toolkit/>

Downloaded 7th Feb 2018.

1.2.6 Effectiveness and global impact of RV1 and RV5

Following introduction of RV1 and RV5 in countries across the world, post-licensure surveillance studies were undertaken to determine impact on rotavirus diarrhoea and mortality. Lamberti et al. (2016) completed a systematic review of all such studies published up to October 2014. Included in the effectiveness meta-analysis were 19 observational studies and one cluster

randomized controlled trial. Studies relating to RV1 and RV5 were grouped together for analysis. Pooled estimates for vaccine effectiveness were generally higher than those for vaccine efficacy, but the disparity between high- and low-income settings remained evident. The pooled estimate of vaccine effectiveness against rotavirus hospitalizations in children under five years old for developed countries was 88.9% (80.9-93.5%), for Latin America and Caribbean countries was 67.6% (54.8-76.7%) and for Sub-Saharan Africa was only 57.0% (40.0%-68.0%).

Three studies in Latin America and Caribbean countries demonstrated a decline in diarrhoeal mortality post vaccine introduction with a pooled reduction of 41.2% (39.9-42.4%) (Richardson et al., 2010, Lanzieri et al., 2011, Bayard et al., 2012). Tate et al. (2016), revising regional estimates of rotavirus mortality in 2013, noted that although vaccines had been available since 2006, early introductions were mostly in low-mortality countries. At that time, four countries, India, Nigeria, Pakistan and Democratic Republic of Congo, accounted for almost half of all rotavirus deaths but had not yet introduced national vaccination. Tate et al. (2016) reflected that the full impact of rotavirus vaccination on diarrhoeal mortality had yet to be realized.

1.2.7 Other rotavirus vaccines

The monovalent ORV-116E rotavirus vaccine ROTAVAC® has recently achieved pre-qualification by WHO, allowing this vaccine to be introduced as an alternative to RV1 and RV5 at the preference of national vaccine programmes. The heat-stable pentavalent bovine reassortant vaccine, ROTASIL®, is currently seeking WHO pre-qualification. Several other vaccines have been locally licensed, and others are in Phase I/II development. These vaccines are summarized in Table 1-1.

Concerns about the efficacy and safety profile of oral, live rotavirus vaccines have led to the development of non-replicating parenteral vaccines as an alternative. Vaccine candidates based on inactivated rotavirus particles, protein sub-units or virus-like particles are currently being investigated in animal models (Kirkwood et al., 2017). A recent Phase I/II trial of the parenteral, non-replicating, monovalent P2-VP8-P[8] sub-unit vaccine in South Africa showed promising results in safety and immunogenicity (Groome et al., 2017).

Table 1-1: Other rotavirus vaccines

Vaccine (manufacturer)	Design	Stage of Development	Comments
WHO pre-qualified vaccines			
ORV-116E, ROTAVAC® (Bharat Biotech, India)	Naturally occurring live attenuated monovalent oral human neonatal G9P[11] strain containing one bovine segment (ORV-116E)	Licensed in India in 2014. WHO pre-qualification January 2018. Vaccine efficacy 56.4% (95%CI 36.6-70.1%) in the first year of life (Bhandari et al., 2014)	Three-dose schedule at 6, 10 and 14 weeks
Locally licensed vaccines			
Lanzhou lamb rotavirus vaccine (Lanzou Institute of Biomedical Products, China)	Live, attenuated monovalent oral vaccine based on a lamb G10P[15] strain	Approved for use within China in 2000. Post-licensure vaccine effectiveness estimated at 72% (95%CI 63-79%) (Cui et al., 2016).	Complex schedule. Not available internationally. Publicly reported efficacy data limited. Never introduced nationally. Superseded by RV1/RV5
Rotavin-M1® (Center for Research and Production of Vaccines and Biologicals, Vietnam)	Novel live attenuated oral monovalent human G1P[8] strain	Licensed in Vietnam in 2014. Comparable to RV1 in safety and immunogenicity studies (Dang et al., 2012).	Two-dose schedule at 6 and 10 weeks Efficacy/effectiveness data awaited.
BRV-PV ROTASIIL® (Serum Institute of India)	Lyophilized, live, attenuated, oral, pentavalent bovine- reassortant vaccine based on G6P[5] backbone and including G1, G2, G3, G4 and G9 expressing strains.	Under review for WHO pre- qualification. Licensed in India 2016. Phase III trials in India (Kulkarni et al., 2017) and Niger (Isanaka et al., 2017). Efficacy against severe gastroenteritis much lower in India (36% (95%CI 11.7- 53.6%) than Niger (66.7% (95%CI 49.9-77.9%).	Three-dose schedule at 6, 10 and 14 weeks. First heat-stable rotavirus vaccine, offering significant advantage in logistics and reduced delivery cost in tropical settings.

Vaccine (manufacturer)	Design	Stage of Development	Comments
Vaccines in Phase I/II development			
RV3-BB (Murdoch Children's Research Institute and Biopharma, Australia)	Live, oral, naturally attenuated monovalent G3P[6] neonatal strain	Phase II studies ongoing. Immunogenicity studies in New Zealand were promising, and results awaited from Indonesia (Bines et al., 2015) Phase IIb study ongoing in Malawi	Proposed three-dose schedule, including a birth dose. Strain reported to replicate well in the presence of high levels of maternal antibody.
P2-VP8-P[8]	Parenteral, non- replicating, monovalent P2-VP8- P[8] sub-unit vaccine	Phase I/II Promising initial safety and immunogenicity study in infants in South Africa (Groome et al., 2017).	First parenteral non- replicating rotavirus vaccine to be studied in infants. May avoid problems associated with oral vaccines.

1.3 POTENTIAL CONTRIBUTORY FACTORS TO REDUCED ROTAVIRUS VACCINE EFFECTIVENESS

1.3.1 Overview of potential contributory factors

Multiple explanations for the rotavirus vaccine efficacy disparity between high- and low-income countries have been proposed (Figure 1-4). These include factors which may reduce the overall host immune response, such as malnutrition or HIV; factors which may inhibit or interfere with the host immune response to vaccination, such as high levels of passively-acquired maternal antibody, concurrent oral polio immunisation, genetic polymorphisms, or concurrent gastro-intestinal infection or inflammation; and factors relating to local viral epidemiology, such as high burden of infection and strain diversity.

To date, there are limited data available to support any of these proposed causes. It is likely that so great a disparity between populations is multi-factorial, but given the huge burden of diarrhoeal disease in low-income countries, the identification and modification of any contributory factor could have significant impact.

It should be noted that lower rotavirus vaccine efficacy was evident in the ideal conditions of vaccine trials. Sub-optimal conditions of vaccine delivery, such as an inefficient cold chain, could further limit vaccine effectiveness in real-world settings. However this would not explain the

underlying differences between populations. Similarly, population differences persist despite high rotavirus vaccine uptake.

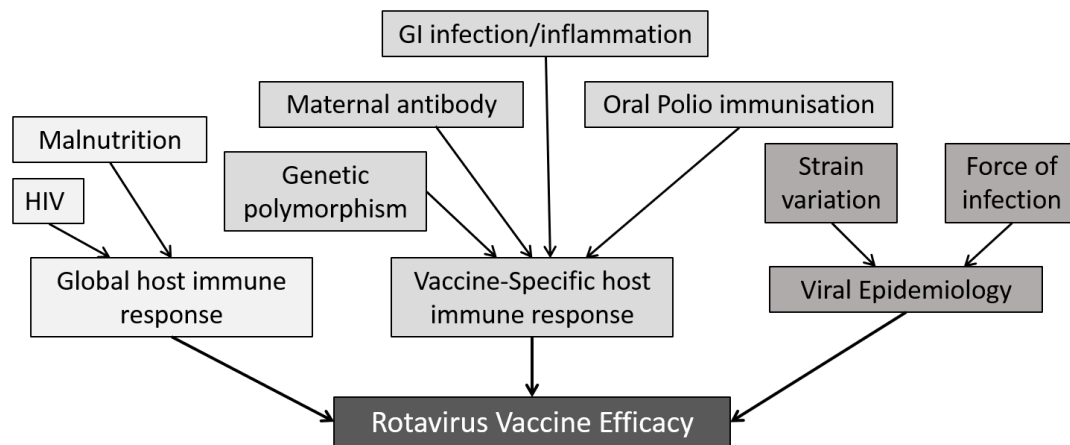


Figure 1-4: Factors proposed to determine rotavirus vaccine efficacy

1.3.2 Factors relating to viral epidemiology

1.3.2.1 Strain variation

Rotavirus strain diversity arises from genetic reassortment, point mutation and interspecies transmission. Global variation in strain distribution and diversity is well recognized, with higher diversity in sub-Saharan Africa (Todd et al., 2010). Early concern that rotavirus vaccines would not be able to generate sufficient heterotypic immunity to protect against diverse vaccine strains led to the hypothesis that geographical differences in rotavirus efficacy might be explained by inadequate local strain coverage. Further to this, there were concerns that selective vaccine pressure from poor heterotypic cover would drive the emergence of certain strains, in a “serotype” replacement phenomenon similar to that observed for pneumococcal vaccines (Weinberger et al., 2011).

Fortunately, there is little evidence to date that this is the case. Both RV1 and RV5 appear to generate broad heterotypic protection. In a systematic review and meta-analysis of strain-specific rotavirus vaccine effectiveness, Leshem et al. (2014) determined that RV1 and RV5 demonstrated similar effectiveness against homotypic and heterotypic strains. Pooled effectiveness of RV1 was 94% (95%CI 80-98%) against homotypic strains and 87% (76-93%) against fully heterotypic strains in high-income countries. Pooled effectiveness of RV1 in

middle-income countries was lower, but remained similar across strains: 59% (36-73%) against homotypic and 47% (28-61%) against fully heterotypic strains. Pooled strain-specific effectiveness for RV5 showed a similar pattern. Sustained predominance of a single strain was not reported in any study.

There is no evidence to date of an excess of “non-vaccine” strains beyond that which would be expected in the normal oscillation of strain distribution (Doro et al., 2014), however cautious monitoring is required, and strain surveillance studies continue globally.

1.3.2.2 Force of Infection

Force of infection is the rate at which susceptible subjects within a population become infected, and is determined by the number of infectious individuals transmitting the pathogen, the degree of contact between individuals to allow transmission, and the infectiousness of the pathogen. In low income countries with a high burden of infected individuals, large population, and overcrowding or environmental conditions contributing to increased transmission, rotavirus force of infection will be high.

In unvaccinated subjects in settings where there is a high force of infection, early and frequent exposure to rotavirus infection will contribute to the development of natural immunity. Consequently, by the end of the first year of life, both unvaccinated and vaccinated subjects may have attained equivalent protection against rotavirus disease. In a placebo-controlled vaccine trial design, this could bias estimates of vaccine efficacy in later infancy toward the null. Since the acquisition of natural immunity will be slower in unvaccinated subjects in high income settings with a lower force of infection, some authors have suggested that this could explain, or at least contribute to, both the apparent efficacy gap between high- and low-income countries and faster “waning” of immunity in low-income countries (Lopman et al., 2012).

However, this explanation does not account for population differences in immunogenicity of rotavirus vaccines, or early vaccine failure observed in vaccine trials in low income settings.

1.3.3 Factors which reduce overall host immune response

1.3.3.1 *HIV*

In 2016, there were an estimated 160,000 new childhood HIV infections, over 80% of these in infants in sub-Saharan Africa (UNICEF, 2017). A much higher number of children are exposed to HIV in utero, but remain uninfected. The prevalence of HIV infection in pregnant women in sub-Saharan Africa is declining, but remains around 5% for the region overall and up to 16% in Southern Africa (Eaton et al., 2014). HIV-infection and exposure has been associated with reduced response to some childhood vaccines (Obaro et al., 2004, Jones et al., 2011). This has led to the suggestion that lower immune response to rotavirus vaccine or secondary vaccine failure in HIV-infected or exposed infants could contribute to lower population vaccine efficacy in low-income countries with a high prevalence of HIV.

However, the evidence that HIV reduces natural or vaccine-induced rotavirus immunity is limited. A review of studies of rotavirus infection in HIV-infected children found no evidence that rotavirus diarrhoea was more frequent or more severe in association with HIV (Steele et al., 2009). An early trial of monovalent RIX4414 showed the vaccine to be safe and immunogenic in HIV-infected infants (Steele et al., 2011). Similarly, a trial of RV5 vaccine in HIV-infected infants showed comparable safety and immunogenicity to HIV-exposed, uninfected infants (Levin et al., 2017). Subgroup analysis of HIV infected infants in published trials of RV1 and RV5 was limited by very small numbers of known HIV-infected infants (Soares-Weiser et al., 2012a) but showed no difference in efficacy.

1.3.3.2 *Malnutrition*

Malnutrition in childhood includes an inter-related spectrum of impairments including intrauterine growth retardation, micronutrient deficiency, stunting and wasting. Childhood malnutrition is associated with poverty and maternal poor health, and is common in low income countries in sub-Saharan Africa and South-East Asia (Black et al., 2013). Reduced rotavirus vaccine response, or secondary vaccine failure, in infants with malnutrition could potentially contribute to low population rotavirus vaccine efficacy in countries with a high prevalence of malnutrition.

Malnutrition is associated with mucosal barrier dysfunction, innate immune dysfunction and thymic atrophy (Rytter et al., 2014). Total leucocyte counts appear unaffected and immunoglobulins are high. Antibody response to most vaccines appear relatively unimpaired, however T cell responses to live vaccines may be sub-optimal (Prendergast, 2015).

There is limited data to demonstrate reduced rotavirus vaccine response in infants with malnutrition. The PROVIDE study in Bangladesh, a large prospective cohort study examining the effect of multiple factors on oral rotavirus responses and child growth and development, found no association between nutritional status at baseline and IgA response to RV1 vaccine (Naylor et al., 2015). Of note, in this study, poor nutritional status at baseline was associated with a reduced response to all other childhood vaccines, except oral polio vaccine. In a sub-group analyses of malnourished children in a trial of RIX4414, Perez-Schael et al. (2007) found no evidence of reduced rotavirus vaccine efficacy. In a post-hoc sub-group analysis of RV5 vaccine studies in Ghana, Kenya, Mali, Bangladesh and Vietnam, Gruber et al. (2017) described a trend toward lower vaccine efficacy in underweight infants in Ghana, but no effect for any other nutritional measure or in any other country studied. Gastanaduy et al. (2016) in Botswana reported that RV1 vaccine effectiveness was significantly higher in infants with no undernutrition, compared to those with moderate or severe undernutrition, however the study was not powered for this sub-group analysis and confidence intervals were wide.

Micronutrient and vitamin deficiencies, particularly of zinc and Vitamin A, have been a focus of recent research interest, given the potential for intervention with supplementation. However, a randomized trial of zinc supplementation in Indian infants showed no benefit in improving rotavirus vaccine immune response (Lazarus et al., 2017). There are no published data on the effect of Vitamin A supplementation on rotavirus vaccine response in humans, however prior trials of Vitamin A supplementation as a method to improve responses to a range of vaccines have been mostly unsuccessful (Savy et al., 2009). Caution is required since harmful as well as beneficial effects of Vitamin A supplementation have been reported, with heterogenous outcomes by population and gender (Jensen et al., 2015).

1.3.4 Factors which reduce specific immune response to rotavirus vaccine

1.3.4.1 *Enteric co-infection and intestinal microbiome at time of immunisation*

In low-income countries, poor sanitation and overcrowding may increase transmission of enteric infection, exposing infants to multiple enteropathogens in early life. Early infections are often asymptomatic, but may affect gut integrity, alter the developing infant microbiome, and activate the mucosal immune system. These effects, together with potential for competitive inhibition, could potentially reduce the replication of and immune response to orally administered live vaccines.

Enteric co-infection at the time of immunisation has been shown to reduce replicative efficiency and immune response to oral poliovirus vaccine (OPV) (Parker et al., 2014), leading to the hypothesis that similar inhibitory effects could contribute to reduced rotavirus vaccine efficacy. However, to date, there is little data to support this hypothesis.

Taniuchi et al. (2016) used quantitative real-time PCR Taqman Array Cards (TAC) to detect a wide range of enteropathogens, including viruses, bacteria and parasites, in Bangladeshi infants at time of RV1 immunisation. Detection of non-polio enteroviruses at time of immunisation was associated with reduced rotavirus-specific IgA response, reduced odds of seroconversion and increased odds of breakthrough rotavirus infection in vaccinated infants. However, no other measured enteropathogen was associated with reduced rotavirus vaccine response.

Parker et al. (2018) used a similar TAC approach to determine the association between enteropathogen burden at time of immunisation and response to RV1 immunisation in infants in India. In this study, enteroviruses were examined in a separate analysis, rotavirus was excluded, and enteroaggregative *Escherichia coli* was considered a commensal organism. In contrast to the initial hypothesis, the presence of more than one enteropathogen present at both doses was associated with increased odds of seroconversion (OR 2.25, 95%CI 1.15-4.41). This association appeared to be attributable primarily to bacterial pathogens, since the prevalence of viral and other pathogens did not differ by vaccine response. Total pathogen count and reported concurrent diarrhoea did not differ between infants who seroconverted and those who did not. There was no association observed between enteropathogen burden at

time of immunisation and rotavirus vaccine virus faecal shedding. The authors reflected that this unexpected positive association between bacterial co-infection and rotavirus vaccine response could have several explanations. Given the potential inhibitory effect of OPV on RV1 vaccine response, suppression of OPV replication by other enteropathogens could offer RV1 vaccine virus a replicative advantage. Alternatively, induction of innate immunity by bacterial pathogens could have created an adjuvant effect to boost RV1 immunogenicity.

Based on similar principles to the hypothesis that concurrent enteric infection could reduce the infant response to oral vaccines, it has been proposed that differences in the infant microbiome between high- and low-income countries could explain population differences in vaccine efficacy. Harris et al. (2017) in a matched case:control study, compared faecal microbiome composition between rotavirus vaccine responders and non-responders in Ghana, and to a control group of age-matched Dutch infants. Total numbers of Ghanaian infants were small (39 responders and 39 non-responders). There was no difference in diversity of the infant microbiome between infants who seroconverted and those who did not. However, the authors reported differing microbiome composition between the groups, with increased abundance of *Streptococcus bovis* and decreased abundance of the Bacteroidetes phylum associated with seroconversion. The microbiome of Ghanaian infants who seroconverted was found to be more similar to that of Dutch infants than those who did not seroconvert. In a questionable approach, Dutch infants did not receive rotavirus vaccine, but were all assumed to be “good responders”.

In common with the Ghanaian study, Parker et al. (2018) in a nested case:control study comparing 85 responders and 84 non-responders, found no relationship between microbiome diversity and seroconversion in Indian children following RV1 immunisation. However, there was some evidence that measures of microbiome abundance were predictive of rotavirus vaccine virus faecal shedding post-immunisation. The relationship between *Streptococcus bovis* and Bacteroidetes and vaccine response observed in Ghana was not reproduced in the Indian study. This could be due to methodological differences or reflect the challenges of determining consistent mechanistic conclusions in complex biological systems.

Of note, the Indian study was a sub-study of a randomized placebo-controlled trial assessing the effect of zinc, probiotics or a combination of both in improving vaccine response. Probiotic administration had little measurable effect on the infant microbiome. Neither zinc nor probiotics alone improved vaccine response, although a combination of the two was associated with a modest increase in seroconversion (Lazarus et al., 2017). Similarly, attempts to improve oral polio vaccine response through use of antibiotics, rather than probiotics, to alter the microbiome have been disappointing (Grassly et al., 2016), despite reducing the burden of enteropathogens.

This highlights that even if the presence of enteropathogens or an unfavourable microbiome are shown more conclusively to inhibit oral rotavirus vaccine response, effective interventions may not be a simple to identify or deliver.

The relationship between the infant microbiome, enteropathogens and oral vaccine response is clearly complex. More data is required to support the hypothesis that this explains differences in vaccine efficacy in different settings. The ongoing RoVi study, will provide further data for infants in England, Malawi and India (Sindhu et al., 2017).

1.3.4.2 Environmental Enteric Dysfunction

Environmental enteric dysfunction (EED), previously termed environmental enteropathy or tropical enteropathy, is a subclinical disorder of the small intestine widespread in low-income settings and evident from early infancy. The condition is characterised by structural changes to the villi reducing gut absorptive capacity, increased small intestinal permeability, resulting in microbial translocation, and chronic mucosal and systemic inflammation. The exact pathogenesis is poorly understood, but recurrent exposure to enteric pathogens may alter the infant microbiome and begin a chronic cycle of reduced gut integrity and immune activation. The condition is strongly associated with poverty and poor sanitation and is most common in tropical countries (Prendergast and Kelly, 2012). There is a long-term association with malnutrition, although the causative relationship between the two conditions is complex and bidirectional.

There is currently no accepted case definition or definitive diagnostic marker of EED. Since the condition is predominantly subclinical, and intestinal biopsy is impractical and unethical in large scale studies, research studies primarily rely on use of faecal and systemic biomarkers of intestinal inflammation (Prendergast et al., 2015).

EED could potentially represent an intestinal environment unfavourable to the replication of orally administered vaccine viruses. In addition, associated mucosal immune dysfunction could limit an effective vaccine immune response. The high prevalence of EED in low-income countries could explain population differences in rotavirus vaccine efficacy.

Several studies have investigated the relationship between biomarkers of EED and rotavirus vaccine response. In a large cohort study in Bangladesh, (Naylor et al., 2015) identified markers of EED in over 80% of infants by 12 weeks of age. In a complex, multivariable analysis, higher levels of alpha-1-antitrypsin, a biomarker of inflammation and epithelial integrity, were associated with lower rotavirus-specific IgA response. No other EED biomarker tested predicted rotavirus vaccine immunogenicity. Higher levels of neopterin, a biomarker of enteric inflammation, were associated with reduced clinical protection from rotavirus diarrhoea, however higher levels of reg1 β , also a biomarker of enteric inflammation, were positively associated with protection against rotavirus diarrhoea. The authors concluded that EED was associated with reduced rotavirus vaccine response and did not comment on this inconsistency.

In a much smaller cohort in Nicaragua, (Becker-Dreps et al., 2017) also reported an association between biomarkers of EED at time of immunisation and reduced seroconversion following RV5 immunisation. Infants who failed to seroconvert had higher levels of the enteric inflammatory markers myeloperoxidase and calprotectin, and a higher median combined score based on four EED biomarkers tested. However, the definition of seroconversion used was a four-fold rise in IgA titres, and infants seropositive (rotavirus specific IgA >20U/mL) at baseline were not excluded. A higher proportion 10/19 (53%) of the infants who failed to seroconvert by this definition were seropositive at baseline, compared to only 3/24 (23%) infants who seroconverted. Seropositivity at baseline suggest prior exposure to wild-type rotavirus, which

may also be associated with reduced seroconversion (Chilengi et al., 2016) and could be an important confounding factor in such a small group.

EED remains an epidemiologically and biologically plausible contributing factor to observed rotavirus vaccine efficacy differences between high and low-income settings. However, larger studies in different populations with clearly characterised definitions of EED are required to confirm this hypothesis.

1.3.4.3 Maternal Antibody

Trans-placentally acquired antibodies help protect infants from infection in the first few months of life, but also influence the development of the infant's own immune response. An inhibitory effect of high levels of maternal antibody on infant vaccine response has been shown for several vaccines, including measles and polio (Albrecht et al., 1977, World Health Organization Collaborative Study Group on Oral Polio Vaccine, 1995). However, this inhibitory effect is not universal, and varies in extent between vaccine types and doses (Siegrist et al., 1998).

There was evidence of an inhibitory effect of maternal antibody on neutralizing antibody response to the early bovine rotavirus vaccine RIT4237 (Cadranet et al., 1987) but there are no other published data on maternal antibody inhibition of older rotavirus vaccines.

Trans-placentally acquired IgG is highest at birth and declines exponentially over the first year of life. Delaying immunisation may reduce interference from maternal IgG, but must be balanced against delaying protection. In early immunogenicity studies of RV1, Steele et al. (2010b) determined that a schedule given at 6 and 10 weeks was less immunogenic (with seroconversion rates of 36-43%) than a schedule given at 10 and 14 weeks, (seroconversion rates 55-61%). The authors speculated that interference by maternal antibody could be one explanation for this effect.

Appaiahgari et al. (2014) demonstrated that rotavirus -specific IgA response to the Indian neonatal vaccine ORV-116E was inversely proportional to rotavirus-specific IgG (maternal IgG) at time of immunisation. In this study, rotavirus-specific IgG present at 8 weeks was assumed to be passively acquired once infants with high rotavirus-specific IgA were excluded. Infants who

seroconverted had a significantly lower baseline rotavirus-specific IgG than those who did not. However, this difference was not seen for infants given a higher dose of vaccine, suggesting higher antigen doses could overcome the inhibitory effect.

Becker-Dreps et al. (2015) in Nicaragua, found that infants who seroconverted following RV5 immunisation had higher maternal rotavirus-specific IgG than those who did not. Maternal serum IgG in this study was directly measured. Of note, although as expected maternal and infant rotavirus-specific IgG were correlated, no significant difference in infant's own serum IgG at time of immunisation was shown between the two groups. This may be due to small numbers (n=44) in this study.

Moon et al. (2016) in South Africa found that a two-fold rise in pre-immunisation rotavirus-specific IgG reduced the odds of RV1 vaccine seroconversion following the 1st vaccine dose by around 26% (adjusted OR 0.74, 0.55-0.99, p=0.044) but did not significantly reduce the odds of seroconversion following the 2nd dose (adjusted OR 0.83, 0.63-1.08, p=0.16). This might suggest that by the second dose in this population, maternal antibody may have declined below an inhibitory threshold. The negative linear correlation between pre-immunisation IgG and IgA post dose 1 (Spearman's $r=-0.28$, $p=0.03$) observed in this study was weak, which may suggest a non-linear threshold effect.

Further evidence for a threshold effect of inhibition by maternal antibody was shown by Armah et al. (2016) in Ghana, who found much higher seroconversion following RV1 immunisation (39%) in infants with pre-immunisation IgG <25th centile, compared to 6% in infants with IgG >75th centile ($p=0.001$). Chilengi et al. (2016) in Zambia reported a similar, although non-significant, trend.

The hypothesis that high levels of maternal antibody contributes to the efficacy disparity between high and low-income settings depends on the assumption that higher force of rotavirus infection in a community will be associated with higher maternal rotavirus-specific IgG and consequently higher infant levels of passively-acquired antibody (Chan et al., 2011). However, there are limited comparative data to confirm that this assumption. In India, Ray et al. (2007) found that women from a lower socio-economic group in India had lower rotavirus-

specific IgG than women from a higher socioeconomic group, despite a high exposure to rotavirus disease. They also noted higher rates of malnutrition in women from the lower socioeconomic group, and speculated that this could suppress maternal rotavirus immunity. Placental transfer of IgG may also be limited by malnutrition, and other maternal co-morbidities such as malaria and HIV (de Moraes-Pinto et al., 1998).

1.3.4.4 Inhibition of rotavirus vaccine response by breast-milk

Several studies provide evidence suggesting both antibody and non-antibody components of breast-milk play a role in protection against natural rotavirus infection, although effects have not always been consistent (see 1.1.5). It has been proposed that breast-feeding, particularly at the time of immunisation, could therefore inhibit rotavirus vaccine virus replication in the infant gut and consequently limit host vaccine response. Experimental studies demonstrated that infectivity of rotavirus vaccine strains was limited by maternal IgA and other factors in breast-milk (Moon et al., 2010, Moon et al., 2013, Glass and Stoll, 1989). However, evidence of a significant impact of breast-feeding on vaccine response in vivo is more limited. Results from epidemiological studies determining the effect of breast-feeding on response to older rotavirus vaccine were equivocal (Glass et al., 1991), and suggested any inhibition was overcome by the 2nd vaccine dose. A more recent study in South Africa found no effect of maternal breast milk IgA levels on infant rotavirus-specific IgA response to RV1 immunisation (Moon et al., 2016).

A recent observational study in Mexico reported reduced vaccine responses in breast-fed infants, but did not report or adjust for other socio-economic differences between breast-fed and non-breast-fed infants (Bautista-Marquez et al., 2016). Infant feeding practices are highly influenced by social factors, which vary widely dependent on context. For example, the expense of formula milk is prohibitive in low income countries, and therefore bottle feeding may be limited to families with higher income, or those where the mother has died. Since socioeconomic factors may correlate with other potential predictors of vaccine response such as malnutrition, gut health and co-infection, failure to adjust for potential confounders is a major limitation of any study relating to breastfeeding.

Logically, based on comparison of breastfeeding rates and vaccine efficacy, any inhibitory effect of breastmilk cannot be either universal or absolute. For example, in Finland, where rotavirus vaccine efficacy was over 90%, around 50% of infants are exclusively breastfed around the time of vaccination. By comparison in Mexico, where vaccine efficacy was around 75%, fewer than 20% of infants are exclusively breastfed around the time of vaccination. This would suggest that breastfeeding as a single factor is not sufficient to explain rotavirus vaccine failure, at least not in all populations. However, it is possible that the inhibitory effect of breastmilk varies between countries due to population differences in antibody and immune factor milk components, or due to interaction between breastfeeding and other inhibitory factors.

Importantly, three large randomized controlled trials of withholding breastfeeding at time of immunization have shown no benefit in improving rotavirus vaccine response (Groome et al., 2014, Ali et al., 2015, Rongsen-Chandola et al., 2014). This does not completely exclude an inhibitory effect of breastfeeding on vaccine virus replication – this could still be mediated by breastfeeding in the days following vaccination. However, these studies have conclusively demonstrated that any such inhibitory effect is not amenable to a feeding intervention. To limit breastfeeding for any more than a few hours would be impractical, unacceptable to mothers, and potentially harmful. The only feasible approach to offset any inhibitory effect of breastfeeding would be to develop parenteral rotavirus vaccines.

1.3.4.5 Concurrent Oral Polio Immunisation

Poliovirus is an enterovirus causing acute flaccid paralysis. Like rotavirus vaccines, oral polio vaccine (OPV) is a live, attenuated, orally administered vaccine. It has been proposed that co-administration could lead to inhibitory interactions within the gut, with direct competition between the two replicating vaccine viruses. This principle has some support from in vitro studies of rotavirus and enterovirus co-infection, which showed suppression of rotavirus infectivity (Wang et al., 2012). There is also proof of principle of vaccine virus interactions from studies of OPV itself. OPV is a trivalent vaccine, comprising three attenuated Sabin poliovirus sub-types. Sabin 2 has been shown to inhibit shedding of the other two sub-types, providing proof of principle of direct vaccine virus competition.

While almost all high-income countries have now changed to inactivated parenteral polio vaccines, live oral polio vaccines are still used in most low-income countries. An inhibitory effect of concurrent oral polio immunisation on response to rotavirus immunisation could therefore contribute to observed efficacy differences between high and low-income settings.

In studies of earlier candidate rotavirus vaccines, the extent of the inhibitory effect of oral polio vaccine on antibody response to rotavirus immunisation varied with vaccine type, dose and timing. Rennels (1996) in a review of early studies of oral rhesus rotavirus vaccine and bovine rotavirus vaccine candidate RIT4237 found some evidence of an inhibitory effect of OPV on rhesus rotavirus vaccine immune response, but noted that this effect could be overcome with multiple dosing and did not result in reduced clinical efficacy.

Steele et al. (2010a) found a trend toward lower rotavirus-specific IgA response in South African children randomized to receive concurrent oral polio immunisation with RIX-4414 compared to those receiving inactivated parenteral polio (IPV) immunisation. Ramani et al. (2016) similarly randomized Indian infants to bivalent OPV or IPV and found seroconversion rates following RV1 immunisation were 15% lower in the OPV group. Zaman et al. (2009) found a trend toward lower rotavirus-specific IgA response and lower rotavirus vaccine viral faecal shedding in infants receiving concurrent oral polio immunisation with RV1, compared to those receiving the two vaccines 15 days apart.

Similar evidence of an inhibitory effect of concurrent OPV was reported by Ciarlet et al. (2008) who randomized infants in four Latin American countries to receive RV5 with concurrent OPV or with OPV staggered by 2-4 weeks. Rotavirus-specific IgA titres post-immunisation were lower in infants receiving concurrent OPV, but overall seroconversion rates were unaffected. Seroconversion was very high in both groups.

In a post-hoc analysis of data from several immunogenicity studies of both RV1 and RV5 in Latin America, Patel et al. (2012) found seroconversion rates were lower (61% (95%CI 54-69%) in studies where rotavirus vaccine was given with concurrent OPV, compared to those where OPV was not given (75% (95%CI 59-87%). However, the overlap in confidence intervals suggests this reported difference was not significant.

Emperador et al. (2016), in Bangladesh, undertook a post hoc analysis of a trial comparing monovalent, bivalent and trivalent OPV. A natural experiment allowed comparison of infants who received concurrent OPV and RV1. No significant difference was observed in RV IgA concentration or seroconversion between infants receiving the three different OPV vaccines. Infants who received OPV on the same day as RV1 were less likely to seroconvert compared to those who received the vaccines at least one day apart (OR 0.50 (0.34-0.75) $p < 0.001$) and had significantly lower RV-specific IgA concentration. The authors also explored the relationship between time between OPV and RV1 vaccine response. Once infants who received the vaccines concurrently were excluded, there was no difference in immunogenicity by time between OPV and RV dose. The authors suggested that this implied an inhibitory effect at an early stage of vaccine virus replication or immune response.

Despite this evidence of reduced immunogenicity, there is little data to demonstrate reduced clinical vaccine efficacy in infants receiving concurrent OPV. Tregnaghi et al. (2011) found similar rotavirus vaccine efficacy in infants in Latin America who received concurrent OPV to infants from a historical control group in a similar population who did not receive OPV.

Within the next decade, the plan for the eradication of polio worldwide aims to phase out OPV and replace it with inactivated parenteral polio vaccine (IPV). However, given the lack of evidence for an inhibitory effect of OPV on rotavirus vaccine efficacy, whether the switch to IPV will improve clinical protection following rotavirus immunisation remains to be seen. A better understanding of the mechanism and effect of current OPV vaccines on rotavirus vaccine response could help predict the impact of change to IPV.

1.3.4.6 Histo-blood group antigen phenotype

Genetic polymorphisms could potentially determine vaccine response or susceptibility to specific rotavirus infection and contribute to population differences in vaccine efficacy. To date, research has focused primarily on polymorphisms in histo-blood group antigens (HBGA).

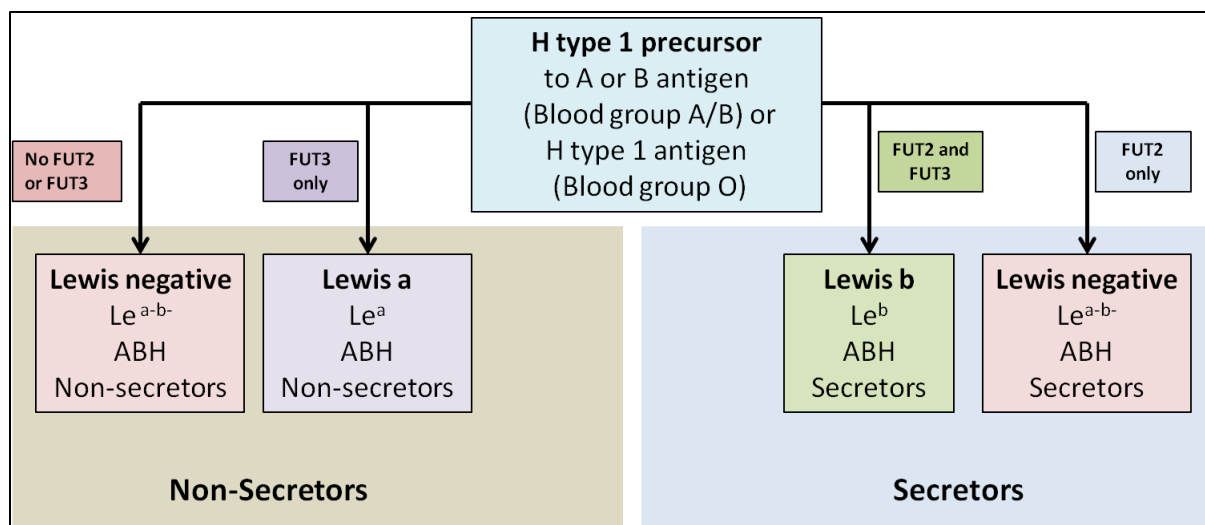


Figure 1-5: Secretor and Lewis Phenotype Pathway

HBGA ABH secretor and Lewis phenotypes are determined by genetic polymorphisms in the expression of glycotransferases (A and B transferases) and fucosyltransferases (FUT2 and FUT3) which catalyse the stepwise addition of monosaccharides to the H Type 1 precursor. Le^b :Lewis b, Le^a : Lewis a, Le^{a-b-} :Lewis negative.

HBGA are complex carbohydrates expressed on the surface of red blood cells and mucosal epithelial cells and as free oligosaccharides in saliva and other exocrine secretions. HBGA oligosaccharide chains are synthesized by the sequential addition of monosaccharides to a disaccharide precursor, with each addition catalysed by specific glycosyltransferases (Figure 1-5). Genetic polymorphisms in the expression of these enzymes determine the final surface antigen phenotype of blood and epithelial cells, and whether antigens are secreted in body fluids. The major ABO blood groups are determined by variation in expression of surface antigens A, B and H Type 1. Secretion of ABH antigens in bodily fluids is determined by expression of the fucosyltransferase FUT2 (Figure 1). Mutations of the FUT2 gene which result in a non-functional enzyme result in the “non-secretor” phenotype. In these individuals ABH antigens are expressed on red blood cells, but not present as free oligosaccharides in exocrine secretions. FUT2, in addition to FUT3, is also a key determinant of Lewis antigen phenotype (Figure 1). Lewis antigens are predominantly synthesised in the gastro-intestinal tract, and expressed on epithelial cells, but also circulate in plasma and are adsorbed to erythrocytes.

In early infancy both the FUT2 and FUT3 transferase enzymes are poorly expressed (Ameno et al., 2001). Lin and Shieh (1994) reported that in Chinese infants of Lewis positive genotypes

FUT3 expression develops initially, followed by FUT2. As a result, genotypic Le^{a-b+} positive secretor infants transition from Le^{a+/b-} to Le^{a+b+} (present in 37% of children age 1-24 months) to a final adult Le^{a-/b+} phenotype by 2 years of age. Morrow et al. (2011) examined FUT2 expression in premature infants, and found that levels of H antigen expressed may also relate to genotype, with higher levels in SeSe homozygotes, and more variable levels in heterozygotes. In effect, it appears many neonates initially have a Lewis negative, non-secretor phenotype, which will gradually evolve over early life according to underlying genotype.

Polymorphisms in human histo-blood group antigens have been shown to confer innate strain-specific resistance to several infectious diseases, including other enteric viruses such as norovirus. In a meta-analysis of genome-wide association studies using data on diarrhoeal disease from several US and European Caucasian cohorts, Bustamante et al. (2016) found a truncating variant in the FUT2 gene to be associated with reduced risk of diarrhoea. HBGA have previously been identified as receptors for norovirus, possibly mediating cell attachment (Tan and Jiang, 2011). HBGA are now proposed to play a similar role in rotavirus pathogenesis.

VP8*, a subunit of the rotavirus major structural protein VP4, is believed to be required for viral attachment to the host cell. Group A rotavirus strains can be grouped into five genogroups (I-V) according to VP8* sequence similarities (Liu et al., 2012). Strains in three groups (I, IV, V) almost exclusively infect animals, group II consist of the major human RVs (P[4], P[6], P[8]), and group III includes strains which infect both humans and animals (P[9], P[14], P[25]). Strains within the same genogroup share common epitopes - sialic acid dependent RVs cluster in group I, while group II and III RVs bind specific HBGA. This could explain differences in host tropism and cross-species transmission.

In vitro studies, including saliva and oligosaccharide binding assays and structural studies, have shown that binding of VP8* to HBGA is strain specific (Table 1-2). Genotypes P[9], P[14], P[25] have been shown to bind HBGA type A (Liu et al., 2012, Hu et al., 2012). VP8* molecules from the closely related rotavirus genotypes P[4] and P[8] have been shown to bind to HBGA Lewis b (Le^b) and H-type 1 (Huang et al., 2012, Ma et al., 2015, Zhang et al., 2016). In contrast genotype P[6], binds H-type 1 only and fails to bind to Le^b positive saliva (Huang et al., 2012, Ma et al.,

2015). Confirming that binding may depend on the interaction of different antigens, in another P[6] binding study, presence of Le^b epitopes blocked binding to H Type 1 (Liu et al., 2016).

Binding may also be age dependent. The expression of HBGA genes develops over infancy, with expression of early precursors preceding full expression of HBGA phenotype. VP8* of the predominantly neonatal P[11] rotavirus strain binds to a precursor of the H glycan expressed primarily in infancy (Ramani et al., 2013, Liu et al., 2013, Hu et al., 2015).

Table 1-2: In-vitro studies of HBGA binding to rotavirus

Study	Methods	VP8* of RV strain	Binding pattern
Liu et al. (2012)	Saliva binding assay	P[9]	Type A HBGA in human saliva
	Haemagglutination assays	P[14]	Bovine and porcine A antigens
		P[25]	Haemagglutination with Type A erythrocytes
Hu et al. (2012)	Glycan array screen	P[14]	Type A HBGA recognized in glycan array screen
	Infectivity assays		Structural basis for binding demonstrated
	Crystallographic studies		Infectivity increased in cells expressing type A HBGA and reduced by anti-A antibodies
Huang et al. (2012)	Saliva, milk and oligosaccharide binding assays	P[4]	P[4] and P[8] bound Le ^b and H type 1 antigens
		P[8]	Binding to Le ^b was stronger in saliva.
		P[6]	P[6] bound H Type 1 antigen only, no binding to Le ^b . Bound saliva of Le ^b negative individuals only.
	Blocking of binding using monoclonal antibodies to HBGA		Binding patterns confirmed by blockade of binding by specific monoclonal antibodies
Ramani et al. (2013)	Glycan array screen	G10P[11]	Precursor of H type II HBGA (LacNAc)
	Haemagglutination assays		H type II glycan increased strength of binding and infectivity of CHO cells
	Infectivity assays on CHO cells		Haemagglutination of adult and cord erythrocytes.
Liu et al. (2013)	Saliva binding assay	P[11]	Binding to saliva in 60.3% infants, no adults
	Glycan array screen		No binding to HBGA (ABH or Lewis)
	Haemagglutination assays		Binding to oligomers of LacNAc
Ma et al. (2015)	Saliva and oligosaccharide binding assays	P[4]	P[4] and P[8] bound Leb and H type 1 antigens and all in secretor ABO types in saliva
		P[8]	
		P[6]	P[6] no obvious binding to ABO positive saliva Weak binding to Le ^b in both saliva and oligosaccharide binding assay. No binding of P[4], P[6] or P[8] to Le ^a or Le ^x

Study	Methods	VP8* of RV strain	Binding pattern
Bohm et al. (2015)	Saturation transfer difference nuclear magnetic resonance	P[8]	P[8] showed no binding to ABH or Leb
		P[9]	P[9] P[14] bound an A epitope independent of histo-blood group specific fucose moiety
		P[14]	
		P[4] P[6]	P[4] P[6] bound A-epitope via fucose moiety, but less strongly than P[14] and P[9] strains.
Zhang et al. (2016)	Saliva binding assay	P[8] P[4]	Binding to Leb+ ABO secretors, but not Leb-Saliva from children with RV infection bound and correlated with host susceptibility Binding weaker to P[4] than P[8]
Sun et al. (2016c)	Saliva and oligosaccharide binding assays	P[19]	Binding to saliva of all HBGA phenotypes No specific binding to any HBGA synthetic oligosaccharides
Liu et al. (2016)	Saliva, human milk, porcine mucin based binding assays Glycan array screening and oligosaccharide binding assays	P[19]	P[19] (a pig rotavirus strain) bound to porcine mucins and human milk, but only to a small proportion of human saliva samples. P[19] P[6] P[8] P[4] all bound to H type 1 antigen P[8] P[4] P[19] bound mucin cores 2,4 and 6 Addition of the Lewis epitope blocked binding of P[19] and P[6] to H Type 1
		P[6]	
		P[4]	
		P[8]	
Sun et al. (2016a)	Saliva-based binding assays Oligosaccharide binding assays Structural analysis of Rotateq VP8*	P[8] VP8* of Rotarix and Rotateq vaccine strains	Binding to ABO secretor saliva, no obvious binding in non-secretors. No specific binding to H type1 or Le ^b in oligosaccharide assay.

However, results from *in vitro* studies have not been entirely consistent, with some variations in binding patterns reported from different studies. Bohm et al. (2015), using structural analysis by nuclear magnetic spectroscopy, reported some evidence of binding to A-type HBGA antigen but not to H type-1 or Le^b antigens. The authors found evidence of binding to A-type HBGA in P[4], P[6] infection, but not to P[8]. Binding of P[14] and P[9] strains to A-Type carbohydrates did not depend on the HBGA-specific fucose moiety. The authors reflected that other glycolipids in epithelial tissues are also fucosylated by FUT2, and therefore the association of HBGA type and strain-specific susceptibility may be indirect. Binding to other HBGA-like complex carbohydrates may be more important. Similarly, Sun et al. 2016a&c failed to demonstrate binding of P[19] or P[8] vaccine strains to HBGA oligosaccharides, but did demonstrate salivary binding. The authors reflected that binding could vary between strains of

the same genotype, that other components in saliva might be required to facilitate binding, or that other similar antigens might be key to binding. These findings suggest interesting alternative hypotheses, but are in direct contrast to other *in vitro* studies and may reflect differences in laboratory technique. Correlation with epidemiological studies is important to provide supportive evidence of clinical significance.

Following from these *in vitro* studies, several clinical studies also suggest that certain HBGA phenotypes may confer resistance to specific rotavirus strains (Table 1-3).

Studies determining the association between HBGA phenotype and rotavirus gastroenteritis in Burkina Faso, Nicaragua, France, Vietnam, USA and China have consistently demonstrated an association between secretor phenotype and increased risk of P[8] rotavirus gastroenteritis (Nordgren et al., 2014, Imbert-Marcille et al., 2014, Trang et al., 2014, Payne et al., 2015, Zhang et al., 2016). Serosurveys in Sweden and China also reported higher levels of P[8]-specific antibodies in secretors compared to non-secretors (Gunaydin et al., 2016, Zhang et al., 2016). In a meta-analysis of four published studies, Kambhampati et al. (2016) reported that overall secretors had a higher susceptibility to rotavirus gastroenteritis compared to non-secretors: OR 4.2 (95%CI 1.1-15.8). This association was strongest for P[8] infections: OR 26.6 (95%CI 8.3-85.0).

Table 1-3: Epidemiological studies of HBGA type and rotavirus

Study	Population	HBGA distribution	Rotavirus genotype distribution	Findings	Comments
Yazgan et al. (2013)	Turkey Retrospective study 288 infants with RV GE 1503 healthy newborn controls	ABO blood group only Controls O 35% A 44% B 14% AB 8%	288 RV GE (no genotype)	No association with ABO group	No secretor or Lewis status
Trang et al. (2014)	Vietnam 260 children with diarrhoea RV test-negative controls	HBGA phenotype All 61% S 12% NS 27% PS	85 RV GE 74 P[8] 5 P[4] 6 P[6]	All P[8] & P[4] RV in secretors or partial secretors. Most expressed Le ^b All P[6] RV infections in secretors, low % Le ^b	Due to small numbers only P[8] results significant by Fisher's exact test. Results not stratified by Lewis status
Imbert-Marcille et al. (2014)	France 57 RV GE cases 95 healthy adult controls 43 non-RV non-NoV GE controls	FUT2 genotype Controls 17% NS	57 RV GE 56 P[8] 1 P[3]	51 RV GE cases FUT2 genotyped, all secretors	No HBGA phenotype No Lewis status
Nordgren et al. (2014)	Burkina Faso and Nicaragua 242 children with diarrhoea Test negative controls in Burkina Faso No controls in Nicaragua	HBGA phenotype FUT3 genotype <i>Burkina Faso</i> n=208 20% NS Leb+54% Lea+14% Lewis negative 32% <i>Nicaragua</i> N=34 6% NS Lewis negative 6%	<i>Burkina Faso</i> 59 RV GE 27 P[8] 27 P[6] 2 P[4] 3 P[6]/P[8] <i>Nicaragua</i> 34 RVGE P[8] 22 P[6] 4 P[4] 7	<i>Burkina Faso</i> All 27 P[8] infections in Le ^b positive secretors 2 P[4] and 3 mixed P[6]/P[8] infections in Le ^b positive secretors Increased odds of P[6] infections in Lewis negative infants OR 5.5 (95%CI 2.3-13.2) <i>Nicaragua</i> All 22 P[8] and all 7 P[4] infections in Le ^b positive secretors 2(50%) P[6] infections in Lewis negative NS	No control group in Nicaragua Study with largest number P[6] infections

Study	Population	HBGA distribution	Rotavirus genotype distribution	Findings	Comments
Ayouni et al. (2015)	Tunisia 114 children with GE RV test negative controls	Phenotype & FUT2 genotype All 21% NS (genotype results similar) Lewis negative 4%	32 RV GE 12 G9P[8] 8 G3P[8] 7 G1P[8] 2 G4P[8] 1 G- P[8] 2 G2P[4]	28/32 RV GE cases secretors 4 non-secretor RV GE cases 1 G3P[8], 3 G9P[8] All RV GE cases in Lewis positive infants (p=0.02) No statistically significant effect of secretor status or ABO	Small numbers Only 4% Lewis negative Test negative controls included 42 NoV positive infants
Payne et al. (2015)	USA 1564 GE 818 healthy frequency age matched controls	FUT2 genotype from saliva Controls NS 23% Lower % of NS (13%) noted in Hispanic controls	189 RVGE 136 G12P[8] 30 G3P[8] 17 G2P[4] 2 G9P[8] 2 G2P- 2 G1P[8]	188/189 RV cases secretors 1 G3P[8] non-secretor 1 vaccinated non-secretor in RV GE group vs 199/1104 (18%) vaccinated controls p<0.001	No Lewis phenotype Limited non P[8] infections
Gunaydin et al. (2016)	Sweden Serosurvey 1008 healthy blood donors and 767 IgA deficiency patients	FUT2 and FUT3 genotyping 21.5% NS Lewis negative 7.2% IgA deficiency group, 9.6% blood donors	Determined RV-specific IgG (n=378) and neutralizing antibodies (n=89) to P[8] and P[6]	RV-specific IgG and neutralizing antibodies to P[8] higher in secretors, and highest in Lewis positive secretors No association with secretor or Lewis status seen for P[6]	Limited P[6] infection in this country
Zhang et al. (2016)	China 266 children with GE 159 healthy controls Test negative controls with NoV excluded Serosurvey 206 healthy volunteers	HBGA phenotype saliva Controls 13% NS 84% Le ^{b+} 13% Le ^{a+} 3% Le ^{a+b+} HBGA phenotype 14% non-secretors 76% Le ^{b+} 14% Le ^{a+} 11% Le ^{a+b+}	75 RV GE 70 P[8] 5 P[4] Determined RV-specific IgG to P[8] & P[4]	All 75 RV GE Le ^{b+} secretors 12 of these were partial secretors Le ^{a+b+} Higher proportion of detectable P[8]IgG and P[4]IgG in secretors and in Le ^{b+} compared to Le ^{a+} or Le ^{a+b+} Lower P[8]/P[4] IgG GMC in non-secretors p<0.001 No difference by ABO groups	

Study	Population	HBGA distribution	Rotavirus genotype distribution	Findings	Comments
Sun et al. (2016b)	China 424 children with diarrhoea RV test negative controls	HBGA phenotype NS 3% S 62% PS 35% Le ^{b+} 79% Le ^{a+} 38%	299 RV GE 277 P[8] G9P[8] 247 G1P[8] 28 G4P[8] 1 P[4] 22	276/277 P[8] infections and all 22 P[4] infections in secretors or partial secretors. 1 non-secretor with G1P[8] Increased odds of RV GE in secretors OR 2.2(1.5-3.3) p<0.001 Reduced odds of RVGE in partial secretors 0.6(0.4-0.9) p=0.01 No difference with ABO	% Non-secretors higher (9%) in previous study the authors in this area in healthy controls. Bias in test negative control group?
Mohanty et al. (2016)	India 147 GE cases RV test negative controls	ABO blood group only All B 36% A 29% O 28% AB 11%	96 RV GE (not genotyped)	21/96 (22%) RV infections in group O infants vs 20/51(39%) group O infants with non-RV GE p=0.02 No other association with ABO	Small number of test negative controls. No secretor or Lewis status
Lee et al. (2018)	Bangladesh Cohort study 275 unvaccinated infants in 1 st year of life 275 vaccinated infants 18 weeks- 1 year	S 66% Le ^{b+} 58% Lewis neg 12% S 69% Lewis neg 17%	104 RV GE episodes P[4] 25 P[6] 9 P[8] 70 47 RV GE episodes P[4] 11 P[6] 7 P[8] 28	Non-secretors decreased risk of any RV GE in 1 st year RR 0.53 (95%CI 0.36-0.79) All P[4] infections in secretors Increased risk of P[6] infection in Lewis negative No significant difference in risk of P[8] or P[6] infection by secretor status No significant association between non-secretor phenotype and vaccine failure RR 0.75 (95%CI 0.41-1.38) Similar trends to unvaccinated infants in RR of P[4] and P[6] infections	

S: secretor, NS: non-secretor, PS: partial secretor, Lewis neg: Lewis negative, RVGE: rotavirus gastroenteritis, RR relative risk, OR odds ratio

Studies which reported Lewis phenotype reported a similar association between Le^b positive phenotype and P[8] infection (Nordgren et al., 2014, Trang et al., 2014, Ayouni et al., 2015, Zhang et al., 2016). However, few studies stratified by Lewis and secretor phenotype. As both Lewis and secretor phenotype are determined by the expression of FUT2, this makes determination of whether expression of one or both antigens contributes to the observed association problematic.

In Burkina Faso, Nordgren et al. (2014), reported that Lewis negative infants had an increased risk of P[6] infection. Trang et al. (2014) in Vietnam also noted lower expression of Le^b in infants with P[6] infection, but numbers were too small for significance. In a Swedish serosurvey, Gunaydin et al. (2016) found no association between secretor or Lewis phenotype and P[6] specific antibody levels, however P[6] rotavirus infection is uncommon in this population. Overall, few studies reported sufficient numbers of P[6] infection for analysis. Meta-analysis of four published studies by Kambhampati et al. (2016) showed no significant association between secretor phenotype and risk of P[6] infection (OR 0.4 (95%CI 0.0-4.1), but association with Lewis phenotype was not reported.

Similarly, few studies reported sufficient numbers of P[4] infection to determine statistically significant associations. In meta-analysis there was no significant association between secretor phenotype and P[4] infection (OR 3.6 (95%CI 0.7-19.6) (Kambhampati et al., 2016). However, almost all reported P[4] infections were in Le^b positive secretors. In addition, in a serosurvey in China, Zhang et al. (2016) found higher P[4] specific antibodies in secretors compared to non-secretors, and in Le^b positive compared to Le^a positive infants. More recently, Lee et al. (2018) reported a significantly increased risk of P[4] rotavirus diarrhoea in secretor infants in Bangladesh in the first year of life.

Two studies, one small study in India (Mohanty et al., 2016) and a larger retrospective study in Turkey (Yazgan et al., 2013), have examined the risk of rotavirus gastroenteritis by ABO phenotype. Yazgan (2013) found no association. Mohanty reported a decreased risk of rotavirus gastroenteritis in Blood group O infants, but numbers were small and the control group potentially biased as it included other diarrhoea cases.

Many studies in this field to date have been limited by small numbers, limited rotavirus strain diversity in some populations, and limited detail in reporting of HBGA phenotype. Some studies have significant methodological limitations. These include the absence of controls, or use of “test negative” non-rotavirus diarrhoea cases as controls. Given the known association between HBGA phenotype and other enteric infections, particularly norovirus, then gastroenteritis controls may be biased and not representative of HBGA phenotype distribution in the general population. Other methodological limitations include retrospective or opportunistic designs. Some studies used genotyping rather than phenotyping to determine HBGA status due to lack of availability of saliva samples. Since the proposed mechanism of association between HBGA and rotavirus susceptibility relies on the expression of HBGA antigens, and full expression of the phenotype is age-dependent, then use of genotyping rather than phenotyping may introduce bias, underestimating the proportion of phenotypic non-secretors in younger infants. However, it could also be argued that HBGA phenotyping by ELISA lacks both sensitivity and specificity compared to genotyping. Variation in laboratory methods used to determine HBGA phenotype and genotype could also contribute to heterogeneity in study results.

Despite these limitations, the sum of evidence to date supports the hypothesis that HBGA phenotype, particularly secretor/Lewis phenotype determines strain-specific susceptibility to rotavirus infection. This may have important implications for vaccine response.

Both current live vaccines include an attenuated P[8] strain. If vaccine strain replication is reduced in Lewis negative or non-secretor recipients, this could limit antigenic stimulus and consequently the host immune response to vaccination. The age-dependent expression of HBGA phenotype (Lin and Shieh, 1994) with poor expression of secretor phenotype in early infancy, could limit response to early rotavirus vaccination with P[8] strains in phenotypic non-secretors. Similarly, genetic polymorphisms in HBGA could potentially contribute to differences in vaccine efficacy. The frequency of the non-secretor phenotype is similar in Caucasian and African populations (20-25%). In contrast, Lewis negative phenotypes are uncommon in Caucasian populations (6-10%), but can be found in up to 40% of individuals in some African

populations. The higher proportion of Lewis negative phenotypes in Africa could contribute to observed differences in vaccine efficacy between sub-Saharan Africa and the US/Europe.

Kazi et al. (2017) explored this hypothesis as a sub-study of a vaccine trial determining the effect of withholding breastfeeding on RV1 vaccine response in Pakistan. HBGA salivary phenotype of subjects in the trial was determined by ELISA. 127/181(70%) infants were secretors and 159/181 (88%) were Lewis positive. Fewer non-secretor infants seroconverted following RV1 immunisation (10/54(19%)) compared to secretors 46/127(36%) (Fisher's exact $p=0.02$). Kazi et al. (2017) further stratified secretor infants by ABO phenotype, and reported that blood group O secretors had increased likelihood of seroconversion compared to non-O blood groups (RR 1.7 (95%CI 1.1-2.7), $p=0.015$). However, numbers in ABO sub-group analysis were small. In those infants who seroconverted, RV-specific IgA concentration did not differ by HBGA phenotype. A higher proportion of Lewis positive infants (41/106(39%)) seroconverted compared to Lewis negative infants (5/21 (24%)), but numbers of Lewis negative infants were small, and this association was not significant (RR 1.6 (95%CI 0.7-3.6), $p=0.25$).

In a cohort study in Nicaragua, Bucardo et al. (2018) reported a different pattern of HBGA-related inhibition of rotavirus vaccine response. The study examined vaccine responses following a single dose of RV5 ($n=68$) and RV1 ($n=168$) immunisation. In this population the proportion of non-secretors was much smaller (around 7%). In pooled results for both vaccines, non-secretor phenotype was not significantly associated with reduced seroconversion (OR 0.57 (95%CI 0.16-2.02). Similarly, no association with seroconversion was found for Lewis b or Lewis negative phenotype. Lewis a phenotype was found to be associated with reduced seroconversion: 0/14(0%) Lewis^{a+b-} infants seroconverted compared to 60/222(27%) of infants with Lewis^{b+} or Lewis negative phenotype ($p=0.02$). There was marginal evidence for an association between B phenotype in secretors and reduced seroconversion following RV1 immunisation: 1/19(5%) type B secretor infants seroconverted, compared to 36/136(26%) seroconversion in type O or A infants ($p=0.046$). No significant association with ABO phenotype was seen when non-secretors were included, or for response to RV5. The study was limited by small numbers in some of the sub-groups, particularly for the RV5 cohort.

Payne et al. (2015) in USA found that non-secretor genotype was associated with reduced likelihood of clinical rotavirus vaccine failure, however the proportion of non-P[8] infection in this population was very small. The study also made no determination of Lewis antigen status.

Lee et al. (2018) reported data from a sub-study of the PROVIDE RV1 vaccine trial in Bangladesh. In the vaccinated cohort, 47/275 infants presented with rotavirus diarrhoea in the first year of life. The risk of vaccine failure did not vary significantly by non-secretor phenotype, although numbers were small (RR 0.75 (95%CI 0.41-1.38). However, the authors found that estimated vaccine efficacy was lower (31.7%) in non-secretors than in secretors (56.2%). They argued that this reflects bias arising from the formula used to calculate vaccine efficacy ($1 - (\text{risk of RVGE in vaccinated infants} / \text{risk of RVGE in unvaccinated infants})$). In this population, in unvaccinated children, non-secretor phenotype is highly protective against rotavirus diarrhoea (RR 0.53 (95%CI 0.36-0.80). Vaccination may therefore add little additional benefit. A low baseline risk therefore results in a misleadingly low estimated vaccine efficacy but low actual risk of clinical vaccine failure in non-secretors. Lee et al. (2018) further hypothesized that in a population with a high prevalence of non-secretors, such as in Bangladesh (over 30%), this effect could lower overall population vaccine efficacy estimates. However, any such effect would not explain observed differences in vaccine efficacy between populations with similar prevalence of non-secretors, e.g. Europe and Africa (both around 20%).

In summary, there is increasing evidence that HBGA, particularly H and Lewis antigens, may be receptors for rotavirus, and that HBGA phenotype may determine strain-specific rotavirus susceptibility and potentially live oral vaccine immune response.

1.3.5 Strategies to improve rotavirus vaccine effectiveness in low-income settings

Multiple factors may therefore contribute to reduced rotavirus vaccine effectiveness in low income countries, and the relative importance and interaction between different factors may vary between and within populations. Understanding rotavirus vaccine responses could better inform understanding of rotavirus epidemiology, pathogenesis and immunity. However, given the ongoing burden of rotavirus mortality, the primary focus should be on factors which are

amenable to intervention. The aim must be to develop strategies to optimize performance of current rotavirus vaccines, and to inform the design and implementation of new vaccines.

Potential intervention strategies to improve rotavirus vaccine effectiveness are summarized in Table 1-4.

Table 1-4: Potential intervention strategies to improve rotavirus vaccine effectiveness

Determinant of reduced vaccine effectiveness	Intervention	Feasibility
Strain variation	Change in vaccine to optimize heterotypic protection	Current vaccines have good heterotypic protection.
Force of Infection	Interventions to improve sanitation and hygiene	Water/sanitation interventions are complex. Rotavirus is endemic even in settings with good sanitation and hygiene
HIV infection/exposure	Interventions to reduce HIV transmission or ensure access to treatment	Many HIV interventions already in place.
Malnutrition	Feeding and food security programmes Micronutrient supplementation	Micronutrient supplementation interventions have shown limited benefit in improving response to other oral vaccines
Enteric co-infection, infant microbiome and environmental enteric dysfunction	Interventions to alter gut bacteria with antibiotics or probiotics New parenteral rotavirus vaccines	Limited benefit in improving response to other oral vaccines. Potential to increase antibiotic resistance Highly feasible if effective. Several vaccines in development Kirkwood, 2017 #1732}
Maternal antibody	Delay vaccine schedule or introduce later booster dose	Some evidence of improved immunogenicity for delayed dosing schedules
Breast-feeding	Withhold breastfeeding at time of immunisation	Several studies show no benefit to this intervention
Concurrent oral polio immunisation	Avoid concurrent administration Introduce inactivated parenteral polio vaccine (IPV)	Avoidance of concurrent administration not logistically feasible. Global switch to IPV planned in next decade, but more expensive and stimulates less mucosal immunity than OPV
HBGA phenotype	Switch to non-P[8] based vaccines eg. RV3-BB, ROTAVAC.	Needs studies comparing newer non-P[8] vaccines to existing vaccines

1.4 PREDICTING ROTAVIRUS VACCINE RESPONSE IN MALAWI: STUDY RATIONALE

This thesis will focus on three main factors potentially determining rotavirus vaccine response: maternal antibody, concurrent oral polio vaccination and HBGA phenotype. These factors were selected as those which are potentially amenable to intervention to improve rotavirus vaccine performance by changes in vaccine scheduling or design.

An infant population in Malawi was chosen to explore the contribution of these factors in predicting rotavirus vaccine virus replication, immune response and clinical protection. Malawi is a low-income country with a well-characterized high rotavirus burden, high rotavirus diversity and low rotavirus vaccine efficacy. It has a well-established national rotavirus vaccine programme with high coverage. It represents an excellent setting for rotavirus research.

Rotavirus in Malawi

Over two decades of rotavirus research studies in Malawi have made an essential contribution to informing local and global strategies to prevent rotavirus disease (Cunliffe et al., 2009a). Initial surveillance studies determined the local burden of disease, defined further by subsequent molecular epidemiology research (Pavone et al., 1990, Turner et al., 2013, Cunliffe et al., 2009b, Cunliffe et al., 2001b, Cunliffe et al., 1999, Cunliffe et al., 2000, Cunliffe et al., 2010, Cunliffe et al., 2001c, Cunliffe et al., 2001a, Cunliffe et al., 2002).

Rotavirus circulates year round in Malawi, with a peak of detection in the cold, dry season (May-October)(Cunliffe et al., 2010). Prior to the introduction of rotavirus vaccine, over 85% of rotavirus gastroenteritis cases attending hospital were in children aged less than one year, 45% were in infants under 6 months and 12% were in infants under 3 months old (Turner et al., 2013). There are few published data on community acquired rotavirus infection in very early infancy in Malawi. In a cross-sectional surveillance study of rotavirus infection in a secondary care neonatal unit in Blantyre found a prevalence of 25%, all with a G8P[6] strain, (Cunliffe et al., 2002). Of note, G8P[6] was found in only 0.5% of older infants from the same community presenting with rotavirus gastroenteritis at that time. It should be noted that risk and exposure to rotavirus infection may vary in a hospital compared to community setting and this data may not be representative of community-acquired neonatal rotavirus infection. Early infant

exposure to rotavirus was estimated at 10% based on rotavirus specific IgA seropositivity at 6 weeks in the Malawi vaccine trial, but this may underestimate infection (Cunliffe et al., 2014).

Most data on rotavirus in Malawi is derived from sentinel surveillance studies. There are no direct cohort data on the incidence of rotavirus infection in young infants. However a pre-vaccine era seroprevalence study of rotavirus-specific IgA in Karonga, rural Malawi, estimated incidence of early rotavirus infection at 0.34 episodes per child year in the first six months of life, and 1.41 episodes per year in the second six months (Bennett et al., 2017).

In common with many other sub-Saharan African countries, rotavirus genotypic diversity in Malawi is high, there is a high proportion of mixed infections, and P[6] infections are more frequently identified than elsewhere in the world (Todd et al., 2010). There is a natural oscillation in rotavirus genotype distribution over time, with emergence of new strains which become transiently dominant, and cyclical re-emergence of some common strains such as G2. In Malawi, G8 strains were emergent in the late 1990's (Cunliffe et al., 1999), G12 and G9 strains in the mid-2000's vaccine trial period (Cunliffe et al., 2012) and G2 strains just prior to vaccine introduction in 2012 (Bar-Zeev et al., 2015). Globally common P[8] strains were dominant in a review of Malawian surveillance data from 1997-2007, accounting for almost half of all strains detected, with high proportion of P[6] strains (19%) identified. At that time, P[4] strains were less common, accounting for around 8% of infections (Cunliffe et al., 2010).

G1P[8] infections have remained continually present, although at a lower rate than globally. In 2013, a novel DS-1-like genetic variant of G1P[8] was identified in Malawi (Jere et al., 2018). This variant differs from previously dominant Wa-like G1P[8] strains similar to the vaccine strain. Similar DS-1-like G1P[8] strains were reported in Asia in 2012-2013 and appear to have arisen through reassortment events with other circulating strains (Yamamoto et al., 2014, Komoto et al., 2016, Nakagomi et al., 2017, Yamamoto et al., 2017). Whether the emergence of novel G1P[8] strains represents natural oscillation or selective vaccine pressure remains unclear.

Surveillance studies established the need for rotavirus vaccines in this setting and informed vaccine development, leading to a major trial of RV1 vaccine (Cunliffe et al., 2012, Madhi et al.,

2010). This trial established that despite efficacy against severe rotavirus gastroenteritis in Malawian infants in the first year of life of only 49%, due to the high burden of disease, RV1 vaccination was highly cost-effective. This led to the introduction of rotavirus immunisation in Malawi in 2012. Results from vaccine effectiveness studies suggest rotavirus vaccine effectiveness in Blantyre is higher than expected, 70.6% (95%CI 33.6-87%) in the first year of life, but still much lower than in high-income settings (Bar-Zeev et al., 2016).

Rotavirus research in Malawi continues through several ongoing studies to assess the impact of vaccine introduction, within the diarrhoeal surveillance platform at Queen Elizabeth Central Hospital, Blantyre. My study was nested within this programme.

1.5 OVERALL HYPOTHESIS

My overall hypothesis was that reduced rotavirus vaccine response in Malawi is multifactorial, and will in part be determined by HBGA phenotype, passively-acquired maternal RV-specific IgG, and oral polio vaccine response. Detailed hypotheses are listed in each chapter.

My aim was to determine which of these three factors, HBGA phenotype, passively-acquired maternal RV-specific IgG and oral polio vaccine response predict vaccine virus replication, immune response and clinical protection in Malawian infants following oral administration of the G1[P8] human rotavirus vaccine (RV1).

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2 GENERAL METHODS

2.1 STUDY CONTEXT

2.1.1 Malawi Demographics and Child Morbidity/Mortality

Malawi is a land-locked country in South-East Africa with an estimated population of 18.6 million people (United Nations Department of Economic and Social Affairs, 2017). Malawi is one of the poorest and least developed countries in the world, with a gross domestic product per capita of only 1,134 international dollars and human development index ranking of 170/188 countries (International Monetary Fund, 2017, United Nations Development Programme, 2016).

Just under 1 in 5 of the Malawian population are children under 5 years old (United Nations Department of Economic and Social Affairs, 2017). Malawi has made significant progress in recent years in reducing child mortality, one of the few countries to achieve the Millennium Development Goal of reducing child mortality by two-thirds between 1990 and 2015 (Kanyuka et al., 2016). However, the under-five mortality rate remains high at 63 deaths per 1000 live births (National Statistical Office (NSO) [Malawi] and ICF, 2017). Two-thirds of deaths in children under five occur in infancy, with an infant mortality rate of 42 deaths per 1000 live births (National Statistical Office (NSO) [Malawi] and ICF, 2017). Over half of these infant deaths occur in the first month of life, mainly due to prematurity or intra-partum causes (National Statistical Office (NSO) [Malawi] and ICF, 2017, Liu et al., 2016). The proportion of infants of low birth weight less than 2.5kg is 12% (National Statistical Office (NSO) [Malawi] and ICF, 2017).

Outside the neonatal period, diarrhoea is a common cause of death, second only to pneumonia, accounting for 11.5% of deaths in young children (Liu et al., 2016). The burden of diarrhoeal disease is high. In 2015, a nationally representative household survey found that 27% of infants less than 1 year old had diarrhoea in the preceding two weeks (National Statistical Office (NSO) [Malawi] and ICF, 2017). In a country where the majority of families live in poverty, the socio-economic impact of diarrhoeal illness may be significant, and may result in delayed presentation to health-care facilities (Masangwi et al., 2016).

Co-morbidities which may increase risk of severe diarrhoeal disease are common. Rates of acute and chronic malnutrition in children are high. Stunting, a marker of chronic malnutrition, is particularly prevalent, with 37% of children under five years old moderately or severely stunted. 12% of children under five years old are underweight for age. Moderate or severe wasting, a sign of acute malnutrition, is found in 3% of children under five years (National Statistical Office (NSO) [Malawi] and ICF, 2017). HIV prevalence in children has declined substantially in recent years, due to successful implementation of programmes to reduce mother to child transmission (Joint United Nations Programme on HIV/AIDS (UNAIDS), 2014). HIV prevalence in Malawian adult women in the Southern Region is 15.7%, with child HIV prevalence estimated at 2-3% (National Statistical Office (NSO) [Malawi] and ICF, 2017, Malawi National Aids Commission, 2015).

2.1.2 Blantyre demographics and local health care

Blantyre District in Southern Malawi has a population of around one million people, over two-thirds of whom live in the City of Blantyre. Poor housing may contribute to risk of diarrhoeal disease locally; 70% of the city population lives in informal settlements with poor sanitation, extremely limited access to running water and significant overcrowding. (United Nations Human Settlements Programme (UN-HABITAT), 2011).

Queen Elizabeth Central Hospital is a tertiary government referral hospital, accepting specialist referrals from throughout the country and providing acute secondary level health-care to a population of around 300,000 children in the City of Blantyre. The paediatric emergency department assesses around 80,000 children per year, with around 25,000 admitted to the hospital. Government health services in Malawi are free at the point of delivery, but resources are limited leading to variable standards of care. Children with acute illness are generally assessed first at primary care health centres, then referred if necessary for hospital admission. There are multiple private primary and secondary health care providers within Blantyre, however private services are unaffordable to the majority of the population.

Prior to the introduction of rotavirus immunisation, rotavirus gastroenteritis accounted for a third of hospitalized cases of gastroenteritis at Queen Elizabeth Central Hospital (Turner et al.,

2013, Cunliffe et al., 2010). Three-quarters of rotavirus cases were in children under 1 year old, with a median age at presentation of 7.8 months (Cunliffe et al., 2010).

2.2 STUDY DESIGN

An overview of study design is detailed in Figure 2-1. A longitudinal cohort design was used to test the hypotheses that HBGA phenotype, passively-acquired IgG, concurrent OPV virus shedding and socio-economic factors would predict viral replication and immune response following RV1 immunisation. A matched cross-sectional case:control design was used to test the hypotheses that HBGA phenotype, RV-specific IgA and socio-economic factors would predict clinical protection following RV1 immunisation.

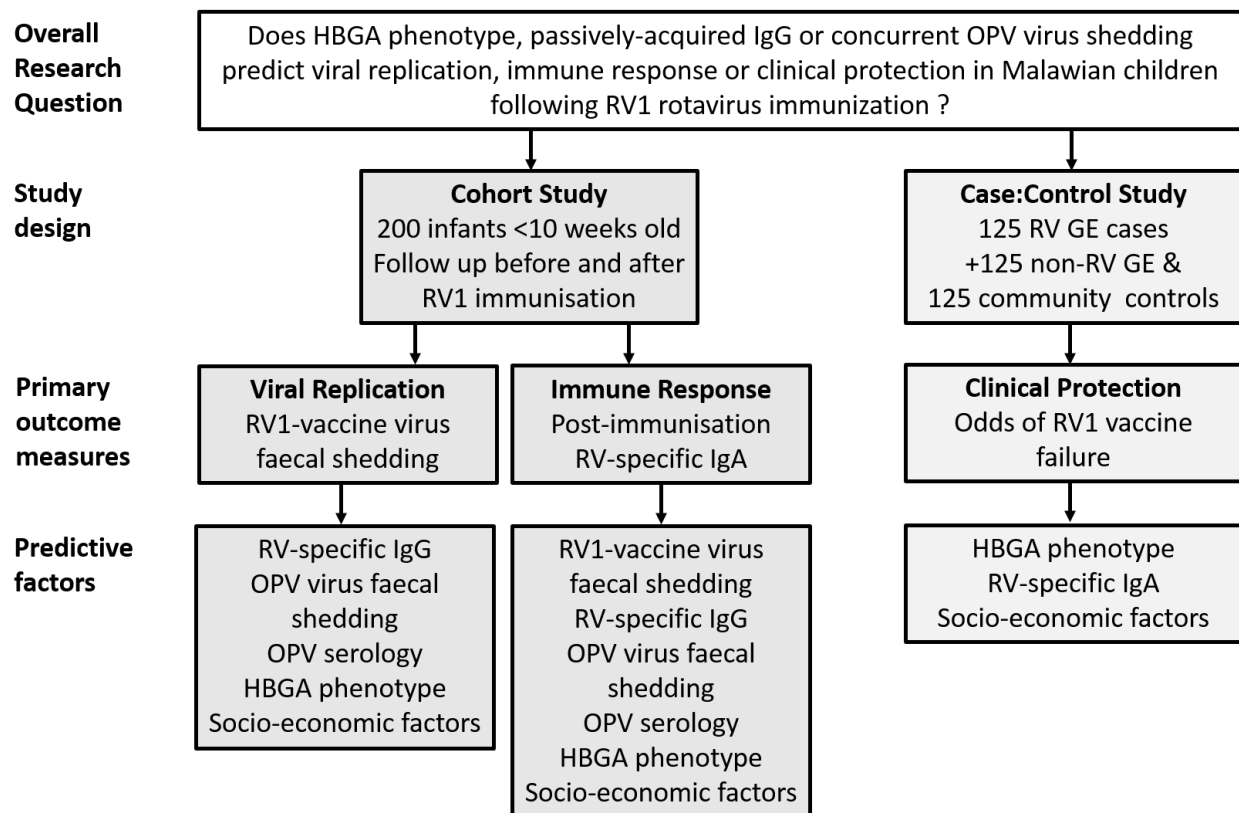


Figure 2-1: Overview of study design

2.3 ETHICAL APPROVAL

Ethical approval was obtained from the University of Malawi College of Medicine Research Ethics Committee (reference P.09/14/1624) and from the University of Liverpool Research Ethics Committee (reference RETH000758). The study was sponsored by the University of Liverpool.

2.4 CONSENT

2.4.1 General principles of consent

Recruitment procedures followed the principles outlined in Good Clinical Practice regarding obtaining and documenting informed consent. Parents/guardians of eligible children who expressed an interest in participating following an initial verbal approach briefly outlining the study were provided more detailed information describing study aims, procedures, potential risks and benefits. This information was provided via written information sheets, and by verbal explanation delivered by research nurses. Both parts of this informed consent process (written and verbal information) were delivered in English or Chichewa in accordance with parent/guardian choice. Questions from parents/guardians were encouraged and answered fully by research nurses. Efforts were taken, within the physical limitations of the study sites, to ensure the consent process was undertaken in an appropriately private environment.

Consent for infants' participation was obtained from parents/guardians who signed, or provided a thumbprint if illiterate, a formal consent form (in English or Chichewa) which made clear the entirely voluntary nature of participation, stating specifically that they could decline to participate, could withdraw from the study at any time, and that non-participation or withdrawal would not impact on any part of the infant's routine healthcare. Where parents/guardians could not provide a written signature, the consent process was observed by an independent witness who verified parental consent and thumbprint by co-signing the consent form. Finally, the consent form was signed by the research nurse or field worker obtaining consent, to confirm the study had been fully explained. A copy of the study information sheet and consent form were offered to the parent/guardian.

2.4.2 Co-recruitment with other studies

Recruitment for the case:control study was integrated with a second separate study aiming to examine factors predicting rotavirus household transmission. Collectively, these studies were referred to as “Rotavirus: Response to immunization and transmission epidemiology” or “RotaRITE”. To maximize scientific benefit, and minimize burden on families, the studies developed integrated recruitment and consent procedures, and shared common demographic and laboratory data.

At QECH, recruitment for both RotaRITE studies was nested within the existing diarrhoeal surveillance platform (VacSurv).

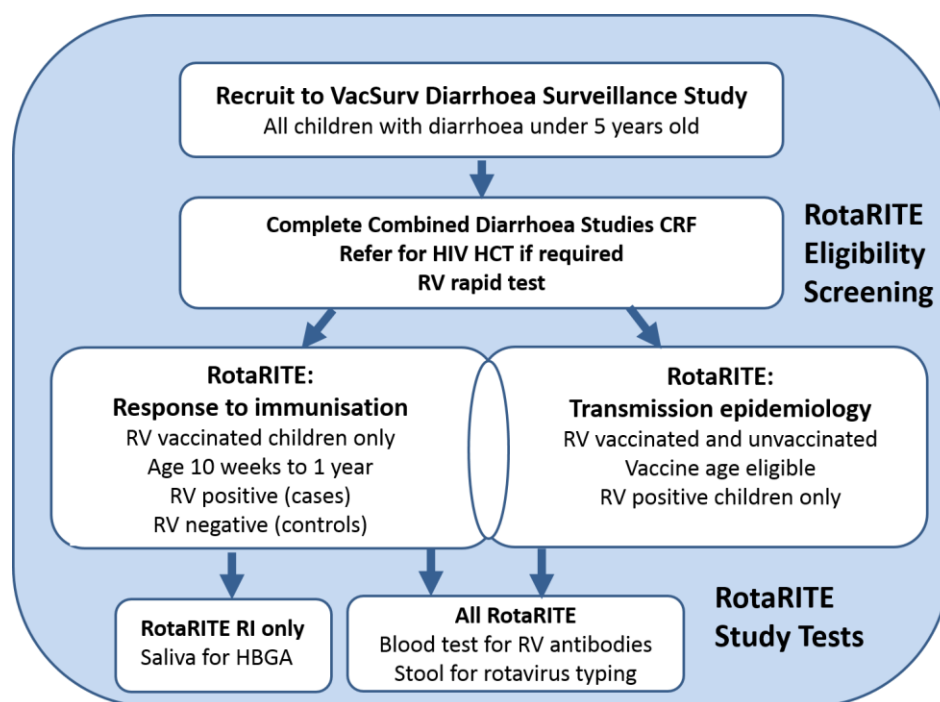


Figure 2-2: Flow chart of integrated recruitment of VacSurv and RotaRITE studies

At QECH, infants and young children presenting with gastroenteritis were first approached to participate in the VacSurv Diarrhoeal Surveillance Study. Within this group, children potentially eligible for the RotaRITE studies were invited to consent to rapid screening for rotavirus, and dependent on the result of this test, invited to participate in one or both RotaRITE studies. Separate information sheets were provided for each study. A common consent form was used. It was emphasized that participation in one study did not imply any obligation to participate in

the others. Specific informed consent to participate in each study, and to share relevant data between the studies, was obtained.

At the health centre study sites, a similar process was used to co-recruit infants to the two RotaRITE studies. Children who presented with diarrhoeal disease were identified directly by study staff. Parents and guardians of these children were invited to consent to an initial eligibility screening using a rapid rotavirus IC test. Depending on the result of this rotavirus test, eligible children were invited to participate in one or both RotaRITE studies, and informed consent was taken for the relevant study.

2.5 RECRUITMENT

2.5.1 Study Sites

Recruitment and follow-up for the cohort study were undertaken at Zingwangwa Health Centre. Zingwangwa Health Centre was selected as a study site for several reasons. Firstly, the health centre had a sufficient number of deliveries per annum to allow recruitment of the required number of infants for the cohort study, and sufficient acute diarrhoea admissions to meet the required number of cases for the case:control study. The health centre had sufficient space to allow research activities to be undertaken without disrupting clinical care. Zingwangwa Health Centre had been used as a site for previous research studies, including the rotavirus vaccine trial, and data from these studies suggested local population demographics were broadly reflective of the wider Blantyre population.

Recruitment of cases for the case:control study was undertaken initially at Queen Elizabeth Central Hospital (QECH) and Zingwangwa Health Centre, Blantyre, Malawi. Due to slower than expected recruitment two further sites were added: Gateway Health Centre and Madziabango Health Centre. Controls were recruited from their homes in Blantyre District. Follow-up for the case:control study was undertaken at a study clinic held in the Paediatric Research Ward, Queen Elizabeth Central Hospital.

Queen Elizabeth Central Hospital was selected as a study site for several reasons. As a referral hospital, QECH had sufficient acute diarrhoea admissions, including severe cases referred from

other centres, to help achieve recruitment targets. Active surveillance of diarrhoea cases was already in progress at this site. There was sufficient space within the Paediatric Accident and Emergency Department to allow research activities to be undertaken without disrupting clinical care. Children are referred to QECH from throughout Blantyre District, so the study population was locally representative.

Gateway Health Centre is a primary care facility situated near to Queen Elizabeth Central Hospital. Madziabango Health Centre is a primary care facility situated in the village of Madziabango, Blantyre District. Gateway and Madziabango Health Centres were chosen as additional study sites as there were a sufficient number of children presenting with gastroenteritis to help achieve recruitment targets. The clinics have sufficient space to allow research activities to be undertaken without disrupting clinical care. Gateway Health Centre has been used as a site for previous research studies and data from these studies suggest local population demographics are broadly reflective of the wider Blantyre population. Madziabango Health Centre was chosen as a population representative of more rural Blantyre District settings.

2.5.2 Cohort study population

Recruitment was undertaken at birth or first postnatal check from Zingwangwa District Health Centre. Infants under 10 weeks old who had not yet received RV1 were recruited. Infants with congenital immunodeficiency, chronic renal or liver disease were excluded.

Families were asked to disclose maternal and infant HIV status. HIV testing was recommended to mothers and infants where HIV status was unknown, in accordance with Malawian national guidelines. Mothers tested during pregnancy were not re-tested, even if tests had been performed more than 3 months before birth. HIV testing was voluntary. Families who declined to disclose HIV status or declined infant or maternal HIV testing were not excluded from the study. Infants with HIV infection were included since evidence suggests HIV status does not affect RV1 response.

Inclusion criteria

- Not yet received any rotavirus vaccine
- Age ≤ 10 weeks old
- Will be resident in Blantyre Municipality for duration of study

Exclusion criteria

- Parent or guardian does not consent or not available to give consent
- Known prior diagnosis of congenital immune deficiency, chronic renal or liver failure or other chronic illness which may affect immune response (excluding HIV)

Baseline social and demographic data were collected by structured interview, performed by a research nurse.

Follow up visits for further data and sample collection took place at the health centre four weeks after the first rotavirus vaccine, and two weeks after the second rotavirus vaccine.

Defaulters were followed up by phone call or home visit. Families received a transport allowance to attend the health centre for immunisation and study follow up. The amount given was based on transport expenses for each participant, as determined by the research field team.

2.5.3 Case:control study population

Children presenting with rotavirus vaccine failure were compared to both asymptomatic community controls, and children with non-rotavirus gastroenteritis. Use of both non-rotavirus gastroenteritis and asymptomatic community controls allowed determination of which factors were specific predictors of rotavirus vaccine failure, and which were non-specific predictors of gastroenteritis. Comparison with community controls also reduced bias toward the null hypothesis arising from over-matching or confounding factors (e.g. the effect of norovirus on distribution of HBGA phenotype) within the non-rotavirus gastroenteritis control group.

Children were recruited in the following groups from Queen Elizabeth Central Hospital (QECH), Gateway Health Centre, Zingwangwa Health Centre, and Madziabango Heath Centre, Blantyre:

- 1. Case-patients:** Children presenting with confirmed severe rotavirus gastroenteritis
- 2. Community controls:** Asymptomatic children matched 1:1 to cases by age
- 3. Non-rotavirus gastroenteritis controls:** Children presenting with confirmed severe rotavirus-negative gastroenteritis, matched 1:1 to cases by age

Inclusion criteria (all cases and controls)

- Age ≥ 10 weeks and ≤ 12 months
- Have received two doses of RV1 (at least two weeks prior to recruitment)

Exclusion criteria (all cases and controls)

- Vaccination status cannot be confirmed
- Resident outside Blantyre.
- Known condition causing immunosuppression (excluding HIV)

Case specific inclusion criteria

- Rotavirus confirmed by rapid immunochromatography test.
- Presenting with severe gastroenteritis (Vesikari score ≥ 11 (Givon-Lavi et al., 2008))
- Duration of gastroenteritis symptoms ≤ 7 days

Non-rotavirus gastroenteritis control specific inclusion criteria

- Born within ± 30 days of matched case
- Recruited within 90 days of presentation of matched case
- Presenting with severe gastroenteritis (Vesikari score ≥ 11)
- Duration of gastroenteritis symptoms ≤ 7 days
- Rotavirus negative on rapid immunochromatography test.

Community control specific inclusion criteria

- Born within ± 30 days of matched case
- Recruited within 90 days of presentation of matched case
- No symptoms of gastroenteritis within one week of recruitment

Control specific exclusion criteria

- Household contact of known rotavirus case

2.5.3.1 Identification and follow-up of cases and non-rotavirus gastroenteritis controls

At QECH cases were identified through the existing active surveillance programme of diarrhoea cases (VacSurv study). Cases were additionally sought by research nurses through ward and hospital admission records. At Zingwangwa Health Centre, Gateway Health Centre, and Madziabango Health Centre, eligible infants were recruited by the health-centre based research nurse through routinely collected admissions data. Cases were consecutively enrolled daily (Monday-Friday, 8-4pm) over the study period.

Non-rotavirus gastroenteritis controls were identified during the screening process for cases. Age-matched controls were recruited as soon as possible following presentation of a rotavirus case. Cases which were unmatched to non-rotavirus gastroenteritis controls 90 days after presentation remained unmatched and were excluded from non-rotavirus gastroenteritis control matched analysis.

For all cases and non-rotavirus gastroenteritis controls baseline social and demographic data was collected by structured interview, performed by a research nurse. Families were asked to disclose maternal and infant HIV status. HIV testing was recommended for acutely unwell infants where status was unknown in accordance with national guidelines. Maternal HIV testing was recommended for all families where maternal HIV status was unknown, in accordance with national guidelines. Families who declined to disclose HIV status or declined infant HIV testing were not excluded from the study. Infants with HIV infection or exposure were not excluded.

Follow-up of cases for further data and sample collection was undertaken 10 days after illness onset at the hospital. Transport expenses were provided. Families who failed to attend follow-up were prompted by telephone or home visit. Cases who did not attend follow-up were not excluded, since the follow-up visit related to a secondary outcome measure and was not essential to primary analysis.

2.5.3.2 Identification and recruitment of Community Controls

Age-matched community controls were recruited from randomly generated locations within healthcare catchment areas specific to each recruitment site. A programme in R 3.1.1 (The R

Foundation for Statistical Computing, code written by Pete Dodd) was used to randomly generate a GPS location within selected Google Earth maps (kml polygons). Data on City of Blantyre healthcare wards was obtained from the Ministry of Health. For children recruited from QECH and Gateway Health Centre a map was created of an area including all City of Blantyre healthcare wards (Figure 2-3).

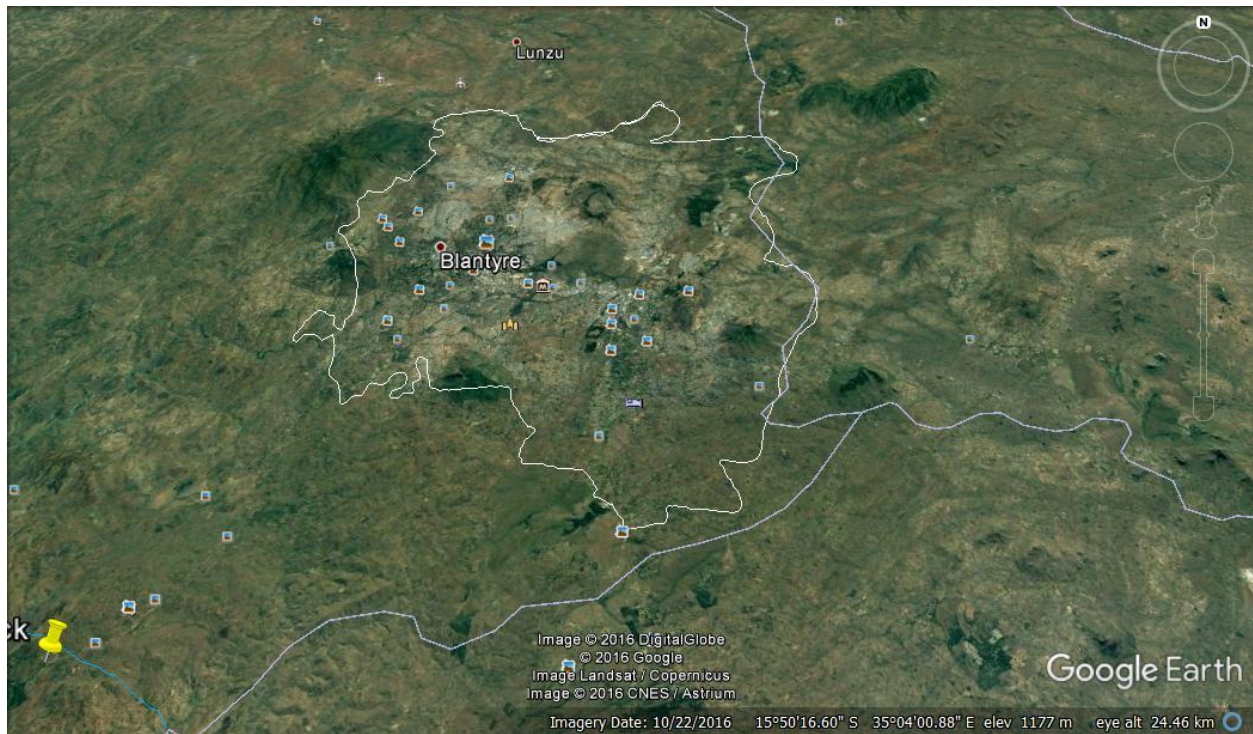


Figure 2-3: City of Blantyre Community Control Recruitment Map

For children recruited in Zingwangwa Health Centre, a map was created of an area including several local healthcare wards from which health centre staff reported their patients attend (Figure 2-4).

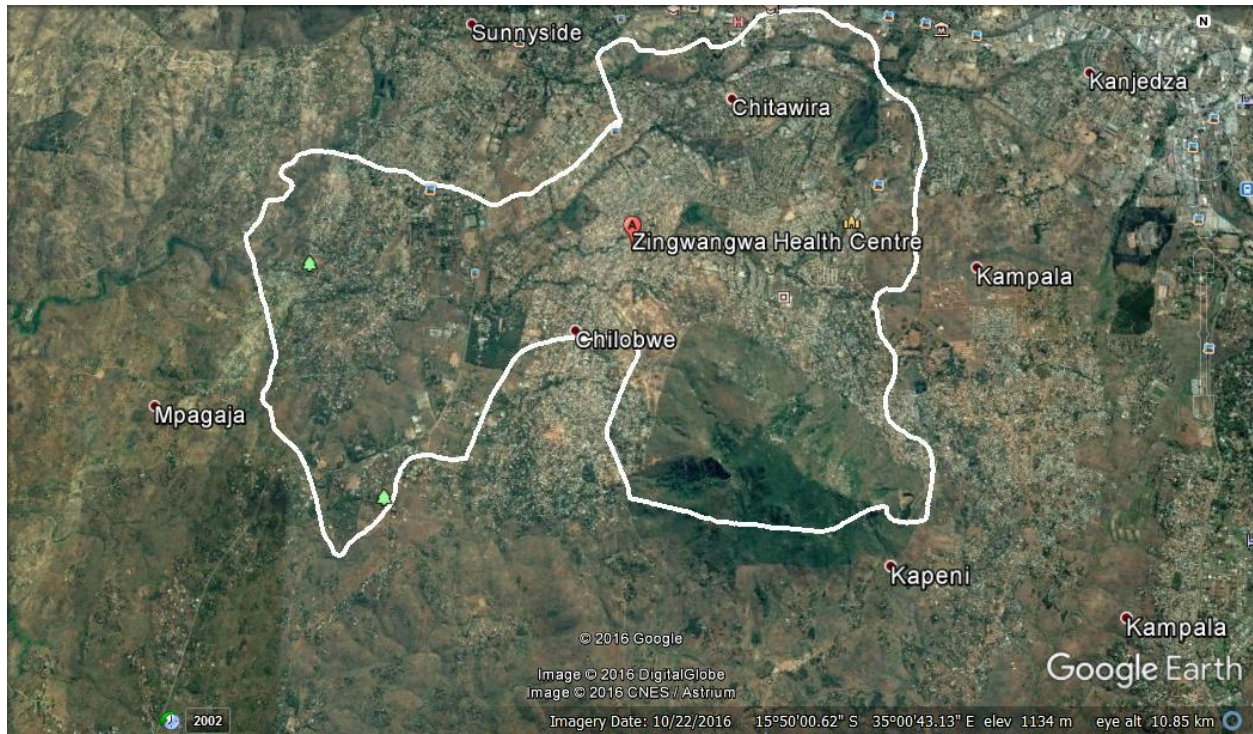


Figure 2-4: Zingwangwa Community Control Recruitment Map

For children recruited from Madziabango Health Centre, since this is a much more rural location, healthcare wards were felt to be less relevant than geographical ease of access. A map was created in consultation with health centre staff of the geographical area from which their patients were reported to attend (Figure 2-5)

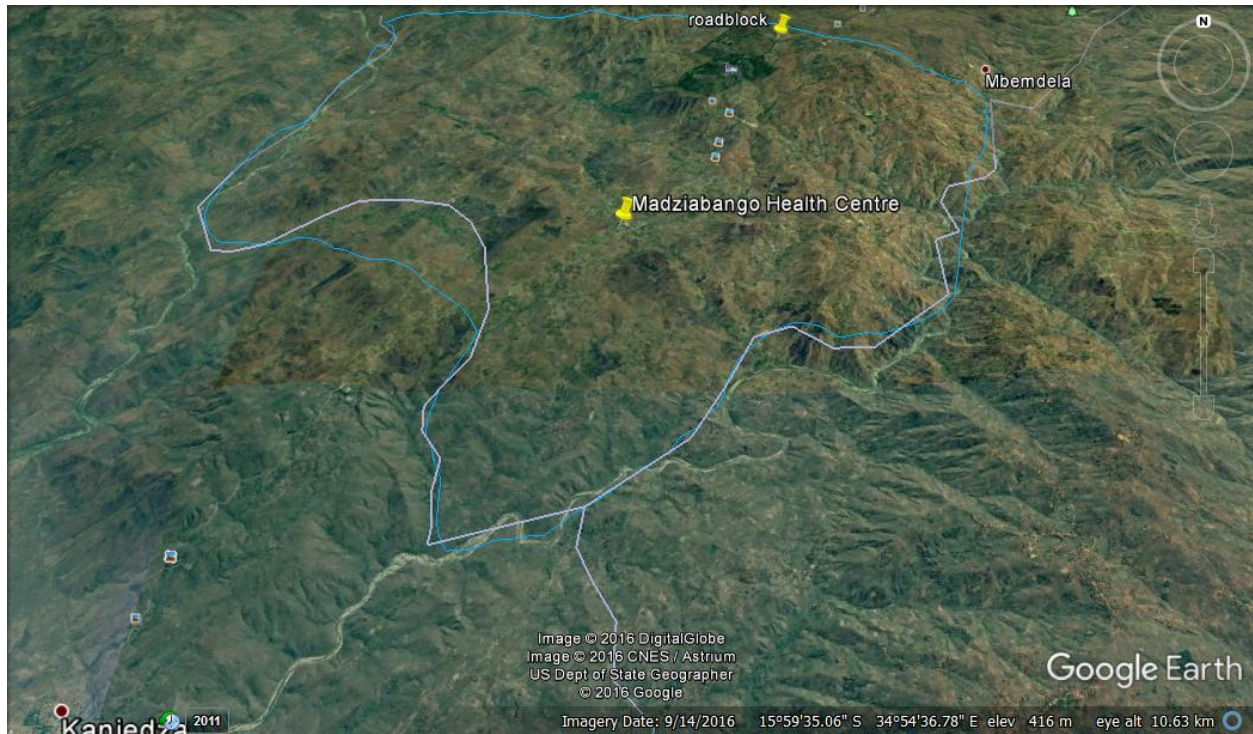


Figure 2-5: Madziabango Community Control Recruitment Map

Once random GPS locations were identified, Google Earth satellite views were used to determine whether the area was inhabited. For safety and logistical reasons, if the area was uninhabited (no dwellings visible within the radius of the random point) this GPS location was excluded, and the next valid GPS point used. Ten random locations were generated at a time, all valid GPS points were visited before a new set of maps were created. Printed maps and directions to the identified point were generated using Google Maps software to aid planning. GPS points were found using Google Maps GPS location software via a Huawei Ascend Y330 smartphone and confirmed with a Garmin etrex 10 GPS receiver. Households nearest the GPS point were visited, moving outwards until suitable control patients were found and initial consent to participate obtained. A maximum of two controls could be recruited from the same location.

Community controls who consented to participate were invited to attend a study clinic held at QECH or the recruiting health centre. Families who failed to attend were prompted by a telephone call or home visit.

2.6 SOCIAL AND DEMOGRAPHIC DATA COLLECTION

Social and demographic data were collected by structured interview. Infant characteristics which could be associated with reduced vaccine response were recorded. Low birth weight, defined as birth weight <2.5kg and determined by verbal report or from the handheld health record was included as a proxy for prematurity, since reporting of gestational age is unreliable in Malawi. Male sex was included as some studies have shown differing vaccine effects according to sex.

Infant and maternal HIV status was determined by verbal report from the carer, confirmed where possible from the child's and mother's hand-held health record. Where status was unknown, mothers were encouraged to attend for routine HIV testing in accordance with Malawi National Guidelines and the result recorded. HIV-exposure was defined as any infant of an HIV-infected mother. HIV DNA PCR test was recommended for all HIV-exposed infants at 6 weeks in accordance with Malawi National HIV Guidelines. HIV DNA PCR tests were performed by Ministry of Health clinical staff and not as part of the study. Results were reported by families to study staff when available.

Key socio-economic data which could potentially be associated with reduced vaccine take were included for analysis. Total household size was included as a proxy for overcrowding. A household was defined as a group of individuals identifying with the same household head. Type of main water source, whether from a piped supply (tap in the home or shared public tap) or non-piped supply (well, borehole or river) was included as a proxy for access to clean water. Time to access water was also included, with higher time assumed to limit access to clean water. Shared toilet with another household was included as a further sanitation indicator. Two proxy measures of poverty, whether there was electricity at home and whether any household member had a salary (defined as regular employment, paid weekly or monthly), were also included. Two questions were asked on food insecurity: "During the last month, how often have you had problems getting the food you need?" and "In the last 2 weeks, has an adult in your household skipped a meal or eaten less in order for there to be enough food?". These variables were combined for analysis purposes as a single variable "food insecurity", defined as

answering “sometimes” or “always” to the first question and yes to the second. Food insecurity was included as both a proxy for poverty, and a risk factor for malnutrition. Finally, level of maternal education was included, as lower maternal education is known to be associated with poverty and poor child health outcomes.

Expected distribution of social and demographic indicators is summarized in Table 2-1.

Table 2-1: Key social and demographic indicators in urban Malawi

Characteristic	Expected distribution in Urban Malawi
Low birth weight < 2.5kg	12% ^a
Sex ratio at birth (M:F)	1.03 ^{b†}
HIV exposure (based on HIV prevalence in women age 15-49)	15.7% ^a
Mean household size	4.3 persons ^a
Non-piped water source	14% ^a
Time taken to access water	
<5minutes	55.8% ^a
5-30 minutes	25.6%
>30 minutes	18.6%
Shared toilet (with another household)	51.1% ^a
Electricity at home	48.7% ^a
Currently employed	Men (age 15-49) 81% ^a
	Women (age 15-49) 63%
Median years completed education (women)	9.1 years ^a
Very low food security	23.1% ^c

Data sources a. (National Statistical Office (NSO) [Malawi] and ICF, 2017) b. (United Nations Department of Economic and Social Affairs, 2017) c. (National Statistical Office (NSO) [Malawi], 2011)

2.7 SUMMARY OF LABORATORY METHODS

An overview and timeline of laboratory methods for the cohort study is detailed in Figure 2-6.

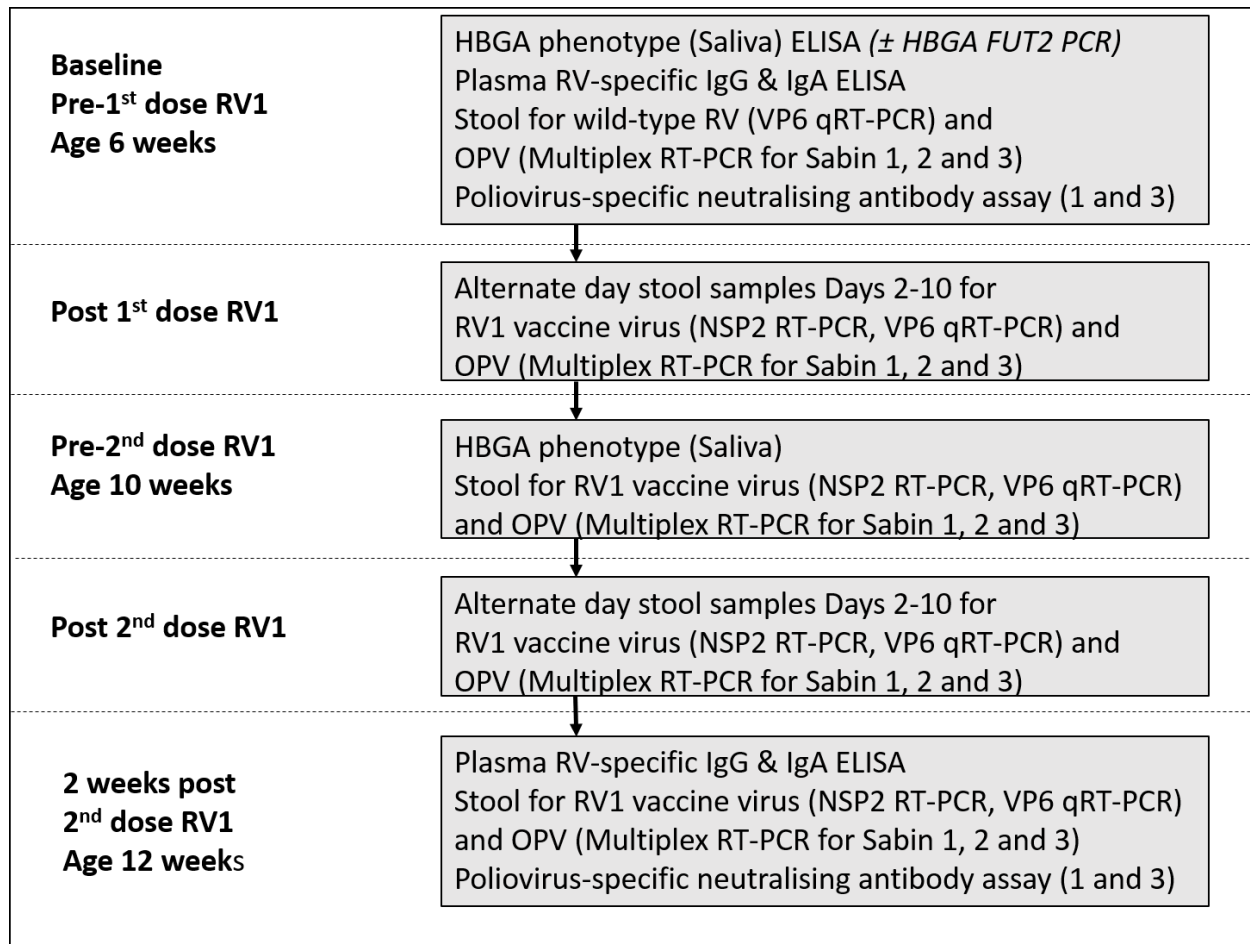


Figure 2-6: Summary of Cohort Laboratory Methods

Laboratory methods for the case control are summarized in Figure 2-7.

Baseline: All cases and controls	HBGA phenotype (Saliva) ELISA (\pm HBGA Fut2 PCR) Stool for RV VP6 qRT-PCR Plasma RV-specific IgA (ELISA)
All cases and RV VP6 PCR positive controls (viral load >100 copies)	RV G and P genotyping by nested PCR and gel electrophoresis
RV cases only	Convalescent RV-specific IgA (ELISA) 10 days post symptom onset

Figure 2-7: Summary of case control laboratory methods

2.8 SPECIMEN COLLECTION

Blood

For the cohort study, 1.5-2mL EDTA blood was collected for rotavirus and poliovirus serology and HBGA FUT2 analysis. Blood samples were taken at recruitment (prior to first rotavirus vaccine) and two weeks after the second rotavirus vaccine.

For the case control study, approximately 1.5-2mL EDTA blood sample was obtained for rotavirus serology and HBGA FUT2 analysis from cases and controls at recruitment. A further 1mL EDTA blood convalescent sample for rotavirus serology was collected from cases only 10 days after illness onset.

All blood samples were collected by research nurses using aseptic technique.

Stool

For the cohort study, parents/guardians were provided with sterile stool collection pots and trained to obtain stool from the infant’s nappy. Stool samples were collected by a field worker/study nurse from the participant’s home, or dropped off at the health centre by parents/guardians according to participants’ preference. Approximately 0.5-2mL stool was collected for all stool tests.

For the case control study, for cases and non-rotavirus gastroenteritis controls, stool was obtained by research nurses from the infant's nappy using a stool collection pot prior to discharge. For potentially eligible children with gastroenteritis, where a bulk stool sample could not be easily obtained, a rectal swab was used as an alternative means to perform the rotavirus rapid diagnostic test.

For community controls, parents/guardians were provided with sterile stool collection pots and instructed to obtain stool from the infant's nappy within 24 hours prior to the child's study clinic visit. Approximately 0.5-2mL stool was obtained for all stool tests.

Samples were placed in a cool bag for transport to the lab as soon as possible after collection.

Saliva

For the cohort study, saliva was collected for HBGA phenotyping prior to the first rotavirus vaccination, and prior to the second rotavirus vaccination. For the case control study, saliva for HBGA phenotyping was obtained from all cases and controls at recruitment.

Saliva was collected using SalivaBio (Salimetrics, UK) infant or children swabs (dependent on the age of the child) in accordance with the manufacturer's instructions. Mothers were asked to avoid breastfeeding or offering other food for ten minutes before saliva collection. One end of the swab was placed under the child's tongue, and kept inside the mouth for 60-90 seconds until the lower third of the swab was saturated. The wet swab was placed into a SalivaBio (Salimetrics, UK) collection tube. Any excess dry swab material was cut off with scissors as required. Samples were placed in a cool bag for transport to the lab as soon as possible after collection.

2.9 INITIAL SAMPLE PROCESSING

Blood

1mL of EDTA blood was transferred to a 2mL cryovial and stored immediately at -80 °C until required for FUT2 analysis. The remaining blood was centrifuged (3000 rpm) at 4°C for 10

minutes to separate the plasma. After centrifugation plasma was pipetted into 2mL polypropylene tubes stored at -80 °C until batch testing.

Stool

Stool samples were stored at 4°C prior to processing. 10-20% faecal suspensions were prepared by adding 200µl (or one pea-sized loop) of fresh stool using a bacteriological loop to 2mLs phosphate-buffered saline in a polypropylene tube. Suspensions were vortexed to create a homogenous solution. The remaining fresh stool was transferred to a 2mL cryovial for storage. For cases also recruited to the VacSurv study, stool suspensions were made similarly, but using RotaClone ready-made buffer for rotavirus enzyme immunoassay (EIA). During EIA testing the sample was centrifuged and 100ul of supernatant used for the test. The remaining supernatant/particulate was vortexed for 30 seconds to resuspend the particulate matter before storage. All fresh stool and suspensions were then stored at -80 °C until batch testing.

Saliva

Saliva samples were processed as soon as possible following collection. In case of unavoidable delay, unprocessed samples were stored at -20°C. Saliva samples were centrifuged in the collection tube at 3500rpm for 15 minutes. One millilitre distilled water was added to the extracted saliva sample. If <50µl saliva was extracted on initial centrifuge, 1mL distilled water was added to the sample swab and centrifuge repeated. Diluted samples were then boiled in a heatblock at 95°C for 10 mins. After cooling, saliva samples were stored at -80 °C until batch testing.

2.10 ROTAVIRUS IMMUNOCHROMATOGRAPHY

Point of care diagnostic testing for rotavirus gastroenteritis was used to identify cases and non-rotavirus controls eligible for the case:control study using the RotaStrip (Coris Bioconcept, Belgium) rapid test. This test uses membrane-based immunochromatography to allow detection of rotavirus antigen. In a positive sample, colloidal gold particles within the nitrocellulose strip bind with Group A rotavirus VP6 proteins present in faecal suspension. The soluble gold/protein conjugate migrates along the strip by passive diffusion, binding to

monoclonal anti-rotavirus antibodies within the test strip, creating a visible red line. A second red line is created at the end of the strip by binding of a control reagent to a control conjugate, confirming the test as technically valid.

The test was performed in accordance with the manufacturer's instructions by research nurses. Using a bacterial loop, 1-2 x10µl of faeces were suspended in 14 drops of the supplied dilution buffer in a test tube and vortexed to ensure a homogenous solution. The test strip was placed in the faecal suspension and read after 10 minutes. The presence of two visible lines confirmed a positive test. Presence of the control line only confirmed a negative test. Tests where the control line was absent were considered invalid and repeated.

The manufacturer reported sensitivity and specificity of the test by third party validation was 98.1% (95% CI 92.7%-99.7%) and 100% (95% CI 95.7%- 100%) respectively using rotavirus enzyme immunoassay as the gold standard.

2.11 ENTERIC VIRUS DETECTION: MOLECULAR LABORATORY METHODS

2.11.1 Molecular Laboratory Work: Quality Control

To minimize the risk of cross-contamination with foreign RNA/DNA, good laboratory practice and training were implemented and nucleic acid extractions was performed in a designated PCR clean environment, following the principles of unidirectional work flow. Bench areas and equipment were cleaned with RNase-away spray. All PCR assays were performed using an ABI PRISM 7500 FAST machine.

Internal Control

An internal control for the extraction process was used with each clinical sample. An RNA template of known quantity (Primer Design Internal Real-time PCR Control kit, Primerdesign Ltd.) was co-purified with the sample RNA. Successful co-purification and real-time PCR for the control RNA confirmed efficient extraction and indicated that PCR inhibitors were not present at high concentration. The internal control real-time PCR was run separately from the rotavirus qRT-PCR, to avoid inhibition. Samples falling outwith the manufacturer's recommended control

RT-PCR target Ct value of 26+/-3 were repeated. The internal control RT-PCR was run according to the manufacturer's recommendations.

Positive and Negative Controls

Positive controls were included in each extraction batch to ensure efficient extraction, reverse transcription and amplification of rotavirus RNA. Positive controls were known rotavirus positive stool-suspensions with Ct value between 30-37. Control samples were prepared in aliquots and results across extraction batches compared to ensure consistency. If the positive control Ct value was above 40 (negative), the batch was repeated. A negative control was included in each extraction batch to identify any contamination in the extraction and PCR process. If the negative control was positive for rotavirus, the batch was repeated.

In each VP6 qRT-PCR batch, a no-template control and wild-type rotavirus positive cDNA control were included. In each NSP2 RT-PCR batch, a no-template control and vaccine-type rotavirus positive cDNA control were included. In each OPV multiplex RT-PCR batch, two OPV cDNA controls, one with high and one low Ct value, were included in each PCR run. The Ct value of all positive controls was recorded and monitored to ensure consistency between runs.

2.11.2 Nucleic acid extraction

Nucleic acid extraction for detection of rotavirus and poliovirus vaccine virus in stool was performed using the Qiagen Viral RNA Mini-Kit (Qiagen) in combination with the Primerdesign Internal RNA Control Kit (Primerdesign), in accordance with the manufacturers' instructions.

The procedure was as follows:

Preparation of Reagents

- 310µl Buffer AVE (Qiagen Kit) was added to the tube containing 310µg lyophilized carrier RNA to obtain a solution of 1µg/l. The carrier RNA was dissolved thoroughly, divided into 2 aliquots, and stored at -20°C. Care was taken to avoid more than three freeze-thaw cycles of the aliquots of carrier RNA.
- Lyophilised internal control template RNA was re-suspended in RNase/DNase free water in accordance with the manufacturer's instructions.

- RNA-AVE solution (5.6µl /sample) and internal control template RNA (4µl/sample) were mixed with Buffer AVL (0.56mL/sample)
- Buffer AW1 and AW2 were prepared with the addition of Ethanol (96-100%) in accordance with manufacturer's instructions.

Extraction Spin Protocol

The extraction protocol was as follows:

- All samples and frozen/cooled buffer mixes were allowed to reach room temperature.
- 560µl of prepared Buffer AVL containing carrier RNA/IC template mix was pipetted into a 1.5mL microcentrifuge tube.
- 140µl of stool suspension was added to the Buffer AVL-carrier RNA-IC template mix and mixed by pulse-vortexing for 15 seconds.
- The sample solution was incubated at room temperature for 10 min
- 560µl of ethanol (96-100%) was added to the sample solution, and mixed by pulse vortexing for 15 seconds.
- 630µl of the sample solution was applied to the spin column (in a 2mL collection tube) then centrifuged at 6000g (8000rpm) for 1 min. The spin column was placed into a clean 2mL collection tube, and the filtrate discarded.
- The spin column was opened and step 6 repeated.
- 500µl of Buffer AW1 was added to the spin column, then centrifuged at 6000g (8000rpm) for 1 min. The spin column was placed into a clean 2mL collection tube, and the filtrate discarded.
- 500µl of Buffer AW2 was added to the spin column, then centrifuged at full speed 20,000g (14,000rpm) for 3 mins.
- The spin column was placed into a clean 2mL collection tube, and the filtrate discarded. The spin column was centrifuged again at full speed for 1 minute.
- The spin column in a was placed into a clean 1.5mL microcentrifuge tube and the filtrate discarded. 40µl of Buffer AVE was added to the spin column. The spin column was

closed and incubated at room temperature for 1 min, then centrifuged at 6000g (8000 rpm) for 1 min to obtain eluted RNA. This final step was repeated to obtain eluted RNA.

- Eluted RNA was stored at -20°C until reverse transcription (within 12-24 hours).

2.11.3 Reverse Transcription

Reverse transcription using random primers was used to generate complementary DNA (cDNA) from the total nucleic acid extracted for amplification. Use of random primers allows generated cDNA to be used for both rotavirus PCR assays and for identification of other enteric viruses (Iturriza-Gomara et al., 1999). Random Primers (Invitrogen 3µg/µl) were reconstituted using nuclease free H₂O in accordance with the manufacturer's instructions. 10X PCR buffer (AB) and 25mM MgCl₂ were stored in aliquots. All reagents were stored at -20°C.

Total nucleic acid extract (40 µl per well) was added to a 96 well PCR plate. The plate was sealed (adhesive seal) and seal heated at 97°C on a heatblock for 2-5 minutes to denature. The plate was then transferred to ice. The mastermix was prepared in accordance with Table 2-2. Reverse transcription mastermix (30 µl) was added to each well, then incubated at room temperature (23± 5°C) for 5 minutes to allow primer annealing. The plate was then incubated at 37°C for 60 minutes to allow reverse transcription. Finally, the plate was heated to 95°C for 2 to 5 minutes to inactivate the reverse transcriptase.

Table 2-2: Mastermix for Reverse Transcription

Reagents	µl /reaction
10X PCR buffer (without MgCl ₂ ; AB)	7
25 mM MgCl ₂ (AB)	14
Random Primers @50 M (Invitrogen/AB)	1
dNTPs (10 mM; AB)	1
M-MLV (200U/µl) (Life Technologies)	2
Nuclease free Water	5
TOTAL	30

2.11.4 Rotavirus VP6 qRT-PCR

Wild-type rotavirus was detected by VP6 qRT-PCR, using primers designed to select and amplify a 379 base pair fragment of the gene encoding the group A rotavirus inner capsid protein (VP6)

(Iturriza Gomara et al., 2002). A fluorophore-labelled target-specific hydrolysis probe was used to allow real-time quantitative detection.

Standards were generated by serial dilutions of a VP6 plasmid stock solution of known concentration. Standards were aliquoted in small volumes to reduce freeze-thaw cycles. A standard curve comprising five standards of 300,000, 30000, 3000, 300 and 30 copies was run in triplicate in each PCR run to allow quantification of viral load. Primer and probe composition is detailed in Table 2-3.

Table 2-3: Rotavirus VP6 qRT-PCR Primer and Probe composition

Primer/Probe	Sequence (5'-3')	Nucleotide Positions
VP6F	GAC GGV GCR ACT ACA TGG T	747-766
VP6R	GTC CAA TTC ATN CCT GGT G	1126-1106
VP6P	^{FAM} CCA CCR AAY ATG ACR CCA GCN GTA ^{MGB}	912-935

References: VP6F, VP6R (Iturriza Gomara et al., 2002) VP6 probe (Gray and Iturriza-Gómara, 2011)

The mastermix was prepared in accordance with Table 2-4. VP6 reaction mix (22.5µl) and 2.5µl cDNA was then added to each well of a 96 well FAST plate. The plate was sealed with optical adhesive covers and centrifuged briefly for 5 seconds. Cycling conditions were as follows: 95°C for 2 minutes, then 40 cycles of 95°C for 15 seconds, 60°C for 1 minute.

Table 2-4: Mastermix for Rotavirus VP6 qRT-PCR

Reagents	µl /reaction
Master Mix (with low Rox)	12.5
VP6 F (@20 pmol/µl)	0.5
VP6 R (@20 pmol/µl)	0.5
VP6 probe (FAM_MGB @ 20µM)	0.25
Nuclease free Water	8.75
TOTAL	22.5

2.11.5 Rotavirus Genotyping

Genotyping was undertaken using methods recommended by the EuroRotaNet surveillance platform (European Rotavirus Surveillance Network (EuroRotaNet), 2017). Classification of human rotavirus strains is based on genotyping of two structural proteins which correspond to

neutralizing antibody responses: VP7, to determine “G-type”, and VP4, to determine “P-type”. The method uses a two-stage PCR technique -first amplifying highly conserved regions of the two structural protein genes, then employing a second-stage multiplex PCR to identify the specific genotype. EuroRotaNet methods are frequently updated to ensure that the natural evolution of circulating strains does not result in failure or inaccuracy in genotyping (Aladin et al., 2010, Iturriza-Gomara et al., 2004).

G-Typing consensus PCR (VP7)

In this first round, specific primers are used to amplify an 884bp conserved region of the VP7 gene (Gomara et al., 2001). Primers used are detailed in Table 2-6.

The first-round G-typing PCR mastermix was prepared in accordance with Table 2-5. VP7 reaction mix (45µl) and 5µl cDNA was then added to each PCR tube. Tubes were briefly centrifuged. Cycling conditions were 94°C for 2 minutes, then 35 cycles of 94°C for 1 minute, 52°C for 1 minute, 72°C for 1 minute. Finally, 72°C for 7 minutes then hold at 15°C.

Table 2-5: Mastermix for G-typing consensus PCR

Reagents	µl /reaction
10X buffer II (Invitrogen)	4.5
50mM MgCl ₂	2.0
dNTPs (10mM)	1.0
Taq polymerase 5U/µl (Invitrogen)	0.2
Primer VP7-F (20µM)	1.0
Primer VP7-R (20µM)	1.0
Nuclease free Water	35.3
TOTAL	45.0

Table 2-6: Composition of primers for RV genotyping

Primer	Sequence (5'-3')	Nucleotide Positions	Product Size
VP7-F	ATG TAT GGT ATT GAA TAT ACC AC	51-71	881bp
VP7-R	AAC TTG CCA TTT TTT CC	914-932	
G1	CAA GTA CTC AAA TCA ATG ATG G	314-335	618bp
G2	CAA TGA TAT TAA CAC ATT TTC TGT G	411-435	521bp
G3	ACG AAC TCA ACA CGA GAG G	250-269	682bp
G4	CGT TTC TGG TGA GGA GTT G	480-499	452bp
G8	TTR TCG CAC CAT TTG TGA AAT	176-198	756bp
G9	CTT GAT GTG ACT AYA AAT AC ^a	757-776	179bp
G10	ATG TCA GAC TAC ARA TAC TGG ^b	666-687	266bp
G12	GGT TAT GTA ATC CGA TGG ACG	548-567	396bp
VP4-F	TAT GCT CCA GTN AAT TGG ^c	132-149	663bp
VP4-R	ATT GCA TTT CTT TCC ATA ATG	775-795	
P[4]	CTA TTG TTA GAG GTT AGA GTC	474-494	483bp
P[6]	TGT TGA TTA GTT GGA TTC AA	259-278	267bp
P[8]	TCT ACT GGR TTR ACN TGC ^{b,c}	339-356	345bp
P[9]	TGA GAC ATG CAA TTG GAC	385-402	391bp
P[10]	ATC ATA GTT AGT AGT CGG	575-594	583bp
P[11]	GTA AAC ATC CAG AAT GTG	305-323	312bp

a. Y= C or T b. R= A or G c. N=A or T or G or C References: VP7F VP7R (Gomara et al., 2001); G1, G2, G4, G9 (Gouvea et al., 1990); G3, G9, P[11] (Iturriza-Gomara et al., 2004); G8, G12 (Aladin et al., 2010) ; VP4F VP4R (Simmonds et al., 2008); P[4], P[6], P[9], P[10] (Gentsch et al., 1992); P[8] (Iturriza-Gomara et al., 2000)

G-Typing Multiplex PCR

The second-round G-Typing PCR mastermix was prepared in accordance with

Table 2-7. VP7 reaction mix (48µl) and 2µl of first round PCR product was then added to each PCR tube. Tubes were briefly centrifuged. Cycling conditions were 94°C for 4 minutes, then 30 cycles of 94°C for 1 minute, 42°C for 2 minutes, 72°C for 1 minute. Finally, 72°C for 7 minutes then hold at 15°C.

Table 2-7: Mastermix for G-Typing Multiplex PCR

Reagents	μl /reaction
10X buffer II (Invitrogen)	4.8
50mM MgCl ₂	2.5
dNTPs (10mM)	1.0
Taq polymerase 5U/μl (Invitrogen)	0.2
Primer VP7-R (20μM)	1.0
Primer G1 (20μM)	1.0
Primer G2 (20μM)	1.0
Primer G3 (20μM)	1.0
Primer G4 (20μM)	1.0
Primer G8 (20μM)	1.0
Primer G9 (20μM)	1.0
Primer G10 (20μM)	1.0
Primer G12 (20μM)	1.0
Nuclease free Water	30.5
TOTAL	48.0

P-Typing consensus PCR (VP4)

The first-round P-Typing PCR mastermix was prepared in accordance with Table 2-8. VP7 reaction mix (45μl) and 5μl cDNA was then added to each PCR tube. Tubes were briefly centrifuged. Cycling conditions were 94°C for 2 minutes, then 35 cycles of 94°C for 1 minute, 50°C for 1 minute, 72°C for 1 minute. Finally, 72°C for 7 minutes then hold at 15°C.

Table 2-8: Mastermix for P-typing consensus PCR

Reagents	μl /reaction
10X buffer II (Invitrogen)	4.5
50mM MgCl ₂	2.5
dNTPs (10mM)	1.0
Taq polymerase 5U/μl (Invitrogen)	0.2
Primer VP4-F (20μM)	1.0
Primer VP4-R (20μM)	1.0
Nuclease free Water	34.8
TOTAL	45.0

P-Typing Multiplex PCR

The second-round P-Typing PCR mastermix was prepared in accordance with Table 2-9.

Reaction mix (48µl) and 2µl of first round PCR product was then added to each PCR tube. Tubes were briefly centrifuged. Cycling conditions were 94°C for 4 minutes, then 30 cycles of 94°C for 1 minute, 45°C for 2 minutes, 72°C for 1 minute. Finally, 72°C for 7 minutes then hold at 15°C.

Table 2-9: Mastermix for P-Typing Multiplex PCR

Reagents	µl /reaction
10X buffer II (Invitrogen)	4.8
50mM MgCl ₂	2.5
dNTPs (10mM)	1.0
Taq polymerase 5U/µl (Invitrogen)	0.2
Primer VP4-F (20µM)	1.0
Primer P[4] (20µM)	1.0
Primer P[6] (20µM)	1.0
Primer P[8] (20µM)	1.0
Primer P[9] (20µM)	1.0
Primer P[10] (20µM)	1.0
Primer P[11] (20µM)	1.0
Nuclease free Water	32.5
TOTAL	48.0

Agarose-gel Electrophoresis

A 2% agarose gel was prepared using 2g of Ultra-pure agarose (Life Technologies) in 100mL 1X TBE, carefully heated in a microwave oven. The cooled gel was poured onto a gel plate with 22-28 slot combs placed until set. 10µl of PCR product was diluted in 10µl of gel loading buffer (Blue/orange loading dye, Promega). Slot combs were removed and 20µl of diluted sample added. A 100bp DNA ladder (O'Gene) was added for comparison. The gel plate was placed in an electrophoresis tank and 1X Tris-borate buffer (TBE) with 5mg/L Ethidium bromide added until level. Electrophoresis was begun at a constant voltage of 150V for 5 minutes, before adding further 1X TBE to fully submerge the gel. Electrophoresis was continued at a constant voltage between 5 and 8 V/cm. Once sufficient separation was achieved, gels were transferred to the UV transilluminator for visualization and identification of DNA fragments.

2.11.6 Rotarix™ vaccine specific (NSP2) RT-PCR

Vaccine virus shedding was determined by RT-PCR, using primers and a probe first developed by Gautam et al. (2014). Through comparison of vaccine and wild-type sequences, Gautam et al. (2014) found the non-structural protein 2 (NSP2) gene to be most dissimilar between Rotarix™ vaccine and wild-type G1P[8] rotavirus strains. Primers were designed to select the 281 base pair nucleotide sequence specific to Rotarix™ strains for cDNA amplification by RT-PCR. Use of a target-specific hydrolysis probe allows real-time semi-quantitative detection. The assay was shown to be highly sensitive and specific to the Rotarix™ vaccine rotavirus strain. The assay detected one test sample which proved on sequencing to be a mixed infection of wild-type G1P[8] and Rotarix™, but no other detection of wild-type G1P[8] was found in 200 test samples. Gautam et al. reported overall assay sensitivity to be 100% and specificity, 99%. The efficiency of the assay was calculated to be 94% with a limit of detection of 2 copies (Gautam et al., 2014). Primers and probes were composed as in Table 2-10.

Table 2-10: Composition of NSP2 RT-PCR Primers and Probe

Primer/Probe	Sequence (5'-3')	Nucleotide Positions
RV1NSP2-F	GAACCTCCTT GAATATAAGA TCACACTGA	546-574
RV1NSP2-R	TTGAAGACGT AAATGCATAC CAATTC	826-801
RV1NSP2-Probe	FAM-TCCAATAGAT TGAAGTCAGT AACGTTTCCA-BHQ1	782-753

Reference: NSP2 F, R and Probe (Gautam et al., 2014)

The mastermix was prepared in accordance with Table 2-11. NSP2 reaction mix (22.5µl) and 2.5µl cDNA was then added to each well of a 96 well FAST plate. The plate was sealed with optical adhesive covers and centrifuged briefly for 5 seconds. Cycling conditions were as follows: 95°C for 2 minutes, then 40 cycles of 95°C for 15 seconds, 60°C for 1 minute.

Table 2-11: Mastermix for NSP2 RT-PCR

Reagents	µl /reaction
Master Mix (with low Rox)	12.5
NSP2F (@20 pmol/µl)	0.5
NSP2 R (@20pmol/µl)	0.5
NSP2 probe (FAM_MGB @ 20uM)	0.25
Nuclease free Water	9.25
TOTAL	23

2.11.7 OPV Multiplex RT-PCR

RT-PCR was used to detect the three attenuated poliovirus strains (Sabin 1, 2 and 3) which comprise the trivalent oral polio vaccine (OPV). Primers and probes, as detailed in Table 2-12, were used to amplify non-overlapping strain-specific sequences. Multiplex RT-PCR assays adapted from these primers and probes have been shown to be highly sensitive and specific in the detection of vaccine-type polioviruses in clinical samples (Giri et al., 2017).

Table 2-12: Composition of OPV primers and probes

Specificity	Primer or probe	Primer or probe sequence (5'→3')	Nucleotide positions
Sabin 1	Sab1/PCR-1	CCACTGGCTTCAGTGTTT	2600-2583
	Sab1/PCR-2	AGGTCAGATGCTTGAAAGC	2505-2523
	Sab1/Probe	CY5-TTGCCGCCCCCACC GTTTCACGGA-BHQ3	2563-2540
Sabin 2	Sab2/PCR-1	CGGCTTTGTGTCAGGCA	2595-2579
	Sab2/PCR-2	CCGTTGAAGGGATTACTAAA	2525-2544
	Sab2/Probe	FAM-ATTGGTTCCCCGACTTCCACCAAT-BHQ1	2550-2572
Sabin 3	Sab3/PCR-1	TTAGTATCAGGTAAGCTATC	2591-2572
	Sab3/PCR-2	AGGGCGCCCTAACTTT	2537-2552
	Sab3/Probe	VIC-TCACTCCCGAAGCAACAG-BHQ2	2554-2571

Reference all primers and probes: (Kilpatrick et al., 2009)

The mastermix was prepared in accordance with Table 2-13. Reaction mix (16 µl) and 4µl cDNA was then added to each well of a 96 well FAST plate. The plate was sealed with optical adhesive covers and centrifuged briefly for 5 seconds. The PCR reaction was performed using an ABI PRISM 7500 FAST machine. Cycling conditions were as follows: 95°C for 2 minutes, then 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, then a final 60°C for 1 minute.

Table 2-13: Polio vaccine multiplex RT-PCR mastermix

Reagents	µl /reaction
Primer Design MM (with low Rox)	10
Sab 1 Primer 1 (20µM)	0.4
Sab 2 Primer 1 (20µM)	0.4
Sab 3 Primer 1 (20µM)	0.4
Sab 1 Primer 2 (20µM)	0.4
Sab 2 Primer 2 (20µM)	0.4
Sab 3 Primer 2 (20µM)	0.4
Sab 1 Probe (10µM)	0.2
Sab 2 Probe (10µM)	0.2
Sab 3 Probe (10µM)	0.2
Nuclease free Water	3.0
TOTAL	16

2.12 ROTAVIRUS SEROLOGY

2.12.1 Rotavirus-specific IgA ELISA

Rotavirus-specific IgA in plasma was determined by an antibody-sandwich enzyme- immunoassay (ELISA). This is a well-established method previously used to detect IgA response to both rotavirus vaccine and natural infection (Ward et al., 1990, Paul et al., 2014, Ramani et al., 2016, Premkumar et al., 2014). The principle of the sandwich ELISA is that rotavirus antigens (Bovine G6P[5] strain WC3 propagated in MA104 cells and prepared as cell culture lysates) are first bound to an ELISA plate coated with a capture antibody (rabbit anti-rotavirus IgG, provided by Christian Medical College, Vellore, India). For comparison, mock-infected MA104 cell lysates are similarly incubated to control for non-specific antigenic binding. Serially diluted plasma samples are then added, and any anti-rotavirus specific IgA present should bind to rotavirus antigen. Rotavirus-specific IgA is then detected using biotin-conjugated rabbit anti-human IgA antibodies, followed by an avidin-biotin-peroxidase complex and a peroxidase substrate. Quantification is determined by comparison of net optical density of clinical samples to a standard curve (standardized plasma), created by serial dilution of pooled serum of known concentration (Paul et al., 2014). The standardized plasma was obtained from Christian Medical College Vellore, and had been validated to allow comparison to standards used by previous international studies. One limitation

of this method is that adult plasma may limit the detection of IgA due to high concentration of IgG which may compete for binding. IgG depletion of samples to counteract this was not feasible due to small sample volume. This method has been widely used in and was chosen to allow better comparison to immunogenicity data from prior RV1 studies including the Malawi rotavirus vaccine trial (Madhi et al., 2012).

Buffers and other solutions used were prepared as detailed in Table 2-14.

Table 2-14: Preparation of solutions for rotavirus specific IgA ELISA

Solution	Preparation	Storage
Coating Buffer: Carbonate Bicarbonate (0.05M) pH 9.6±0.2	Carbonate Bicarbonate buffer capsule (C3041, Sigma-Aldrich) 1 capsule/100mL ddH ₂ O	Autoclaved and stored at 4-8°C for up to 2 months
Wash Buffer: 1X PBS-Tween	Phosphate Buffered Saline Tablets (BR0014G, Oxoid) 1 tablet/100mL ddH ₂ O Tween 20% (P1379, Sigma-Aldrich) 0.1mL/100mL ddH ₂ O	Made daily. Stored if necessary at room temperature for up to 2 months.
Blocking/Dilution Buffer 1% Skimmed-milk in PBS-T	0.5g of skimmed milk powder (DairyBell) in 50mL 1XPBS-T	Prepared fresh before use
Citric Acid Phosphate Buffer (0.1M) pH 5.0±0.2	Dissolve 7.23g of citric acid monohydrate and 9.46g sodium phosphate dibasic in 800mL ddH ₂ O. Volume adjusted to pH up to 1 litre with ddH ₂ O*	Autoclaved and stored at 4-8°C for up to 2 months
Stop solution Sulphuric Acid (1M)	27mL concentrated 98% Sulfuric Acid was carefully diluted with ddH ₂ O to a final volume of 500mL	Stored at room temperature for up to 2 months

*ddH₂O= distilled de-ionised water. *Further pH adjustments were made if necessary with gradual addition of sodium hydroxide.*

Procedure: Day 1

A 1:1500 dilution of rabbit anti-rotavirus IgG in Carbonate-Bicarbonate 0.05M coating buffer (pH 9.6 ±0.2) was prepared. 100µl of diluted rabbit anti-rotavirus IgG was added to each well of each of a 96-well plate (Corning Costar ELISA plate) and incubated overnight at 2-8°C.

Procedure: Day 2

- Test plates were washed five times with wash buffer using a Biotek automated plate washer.
- Pre-optimized 1:4 dilutions of MA104 control cell lysate and WC3 virus lysate in 1% skim milk buffer were prepared. Diluted MA104 control cell lysate (50µl) was added to each well of alternate columns of the test plate. Diluted WC3 virus lysate (50µl) was added to each well of the remaining columns.
- Test plates were incubated at 37°C for 60±10min with shaking/rotating (200-250rpm)
- Serial dilutions of standards and clinical samples in 1% skim milk buffer were prepared in a dilution plate.
- Eight 2-fold dilutions of IgA standard were made, with a starting dilution of 1:80. High, low, medium and negative IgA controls were diluted 1:160. Four 2-fold dilutions of clinical test samples were made with a starting dilution of 1:20.
- Test plates were washed five times.
- IgA standard curve (STD IgA) dilutions, diluted HC, MC, LC and NC and of diluted test samples (50µl of each) were transferred from the dilution plate to the test plate.
- Test plates were incubated at 37°C for 60±10min with shaking/rotating (200-250rpm)
- Test plates were washed five times.
- A 1:3000 dilution of biotinylated rabbit anti-human IgA in 1% skim milk buffer was prepared shortly before use. Diluted biotinylated rabbit anti-human IgA (50µl) was added to each well of the test plate.
- Test plates were incubated at 37°C for 30±10min with shaking/rotating (200-250rpm)
- Avidin-biotin peroxidase (Vectastain) was prepared 30 minutes before use. Solutions A and B were mixed 1:1 then diluted 1:1000 in 1X PBS-T.
- Test plates were washed five times.
- Diluted avidin-biotin-peroxidase complex (50 µl) was added to each well.
- Test plates were incubated at room temperature for 30±10min.

- OPD substrate was prepared 30 minutes before use by dissolving one 15mg tablet of OPD in 30mLs 0.1M Citric Acid Phosphate buffer (pH 5.0 \pm 0.2), then adding 10 μ l 30% hydrogen peroxide solution.
- Test plates were washed five times in wash buffer, then once with Citric Acid Phosphate buffer (200 μ l per well).
- OPD substrate (50 μ l) was added to each well
- Test plates were incubated at room temperature for 30 \pm 10min in the dark
- 1M Sulphuric Acid (100 μ l) stop solution was added to each well
- Plates were read at 490nm using the Biotek microplate reader.

Controls, validity criteria and interpretation of results

MA104 “blank” OD values for each sample were subtracted from WC3 values. The standard curve was modelled using four parameter logistic regression (using online software at www.myassay.com). A minimum of 5 valid points was required to generate the standard curve.

For each assay, values for the high, medium and low controls were compared to expected ranges. The negative control should be below <7.0 U/mL. Assays where controls failed were repeated. Results between 7 and 20U/ml may be unreliable. Seropositivity is defined by the RV1 vaccine manufacturer as >20U/mL.

For a sample titre to be considered valid, results from two of the four dilutions had to fall within quantifiable range of the curve with a co-efficient of variation (%CV) \leq 20%. Where necessary, masking of up to 2 consecutive dilutions was used to obtain a %CV of \leq 20%. The final value was the geometric mean of the included dilutions. Where all sample dilutions fell below the quantifiable range, the titre was reported as below limit of quantification. For low titre samples where only the first dilution was within quantifiable range, samples were repeated. For high titre samples where a %CV \leq 20% could not be obtained due to the prozone effect, samples were retested at higher dilutions. Where validity criteria could still not be obtained after retesting, the sample was reported as indeterminate.

2.12.2 Rotavirus-specific IgG ELISA

Methods for the rotavirus-specific IgG ELISA were the same as those for IgA, with the following differences:

- Eight 2-fold dilutions of standard plasma were made, with a starting dilution of 1:100. Four 2-fold dilutions of clinical test samples were made with a starting dilution of 1:100.
- An internal reference sample diluted at 1:1000 (with an expected value of 5000U/mL) was run in duplicate, rather than high, medium and low controls.
- Biotinylated goat anti-human IgG (Vector Labs), rather than rabbit anti-human IgA, was used at a dilution of 1:4000.

2.13 POLIOVIRUS-SPECIFIC NEUTRALIZING ANTIBODY ASSAYS

For safety reasons, all poliovirus serology was undertaken at the Christian Medical College, Vellore, India. In brief, poliovirus-specific neutralizing antibodies were tested by modified micro-neutralization assay in accordance with WHO guidelines (World Health Organization, 1997, Giri et al., 2018). Pre and post immunisation plasma samples at 6 and 12 weeks were tested for poliovirus type 1 and 3 neutralizing antibodies in two-fold serial dilutions from 1:8 to 1:1024. Poliovirus-specific neutralizing antibody responses to serotype 2 could not be tested due to the ban on use of live Sabin 2 viruses in laboratories following the global switch to bivalent OPV in April 2016. Protective titres (seropositivity) were defined as an antibody titre ≥ 8 . This definition (and terminology) is consistent with WHO agreed international standards for polio serology. Seroconversion between 6 and 12 weeks was defined as either a change from seronegative to seropositive, or a four-fold rise in antibody titre for those seropositive at baseline.

2.14 HBGA PHENOTYPING

The genetic basis of HBGA phenotype is detailed in Chapter 1. A, B, H and Lectin antigens should be present in saliva of all secretors, Lewis b may or may not be present. A, B, H and Lectin antigens should be undetectable in saliva of non-secretors, Lewis a may or may not be

present. Partial or weak expression of A, B or H antigens is more common in infants and is generally associated with expression of both Lewis a and Lewis b antigens. Due to the age-dependent development of Lewis and secretor phenotypes (see 1.3.4.6) HBGA salivary phenotype was determined prior to both the first and second dose in cohort infants.

HBGA phenotyping was determined first by detection of antigens A, B, H, and Lewis a and b in saliva by ELISA, using monoclonal antibodies specific to the A, B, H and Lewis antigens, detected by peroxidase conjugated anti-IgM. Participants with clear positive detection of A, B, or H antigens were classed as phenotypic secretors. Infants with saliva positive for either Lewis a or b antigen were classed as Lewis positive, and those negative for both Lewis antigens as Lewis negative. Due to the age-dependent development of Lewis and secretor phenotype, it is recognised that some genotypic secretor infants may express the Le^{a+b+} phenotype in saliva before expression of detectable levels ABH antigens which would determine phenotypic secretor status (Ameno et al., 2001). It was also recognised that secretors can have low levels of detectable Lewis a antigen in saliva, and that in addition there could be some cross-reactivity between Lewis a and b ELISA detection (Piner and Sanger, 1980). Therefore, secretor status was defined primarily by ABH detection, rather than Lewis b phenotype.

Where detection of A, B and H antigens was negative or borderline, secretor and Lewis status were confirmed by ELISAs to detect lectin antigen. Peroxidase-conjugated lectin from *Ulex europaeus* (*Ulex europaeus* agglutinin, UEA-1), allows specific detection of Fuc α 1-2Gal-R present in secretor but not non-secretor saliva. Criteria for classification based on these tests is detailed in Table 2-15.

All saliva samples were diluted 1/1000. No quantification of carbohydrate concentration was performed, however since the assays were not quantitative the only limitation of this approach is in the possibility of false negative results. Infants with a partial secretor phenotype (Le^{a+b+}) well recognised in infants, and common in some populations, could also express lower levels of ABH antigens which could lead to false negative phenotype results. This limitation was addressed by repeating borderline or indeterminate samples. All non-secretors and samples which remained indeterminate on repeat proceeded to HBGA FUT2 genotyping.

It is acknowledged that gene sequencing would have been a more sensitive method of confirmation of Lewis and secretor genotype, but this could not be undertaken within Malawi due to ethical constraints and limited local technical capacity.

Table 2-15: HBGA phenotyping criteria

A, B, H	Lewis b	Lewis a	Lectin	HBGA Phenotype
At least 1 positive	Positive	Negative	Not required	Secretor Lewis positive
At least 1 positive	Negative	Negative	Not required	Secretor Lewis negative
At least 1 positive	Positive	Positive	Positive/weak positive	Partial secretor Lewis positive
All negative	Positive or negative	Positive	Negative	Non-secretor Lewis positive
All negative	Negative	Negative	Negative	Non-secretor Lewis negative
All negative	Positive or negative	Positive or negative	Positive	Indeterminate: Repeat

Optimisation of HBGA Phenotyping ELISA

The method described by Nordgren et al was used as a starting point for ELISA optimization (Nordgren et al., 2013, Nordgren, 2013) . Various monoclonal antibodies were tested for sensitivity and specificity. As there were no manufacturer’s instructions for use of monoclonal antibodies for this specific purpose, antibody concentrations were determined by titration and optimisation. Bovine serum albumin was used as a blocking/dilution solution initially, but resulted in high levels of background when clinical samples were tested, possibly due to the presence of similar carbohydrate antigens from breast-milk in saliva. 1% skim milk was identified as a more efficient blocking solution. Antibody dilutions were titrated and optimised to minimise false positives secondary to high background.

ABO and Lewis Phenotyping ELISA

Carbonate bicarbonate and 1% skim milk buffers were prepared as in Table 2-14. A lower concentration of PBS-Tween was buffer was used. TMB was supplied ready for use.

Procedure Day 1

Saliva samples were diluted 1:1000 in 0.05M Carbonate Bicarbonate coating buffer (pH 9.6 \pm 0.2). 100 μ l of diluted saliva was added to each well of a 96 well plate (Nunc-Microsorp) and incubated overnight at 4°C. For blanks and negative controls, 100 μ l of coating buffer was added. Separate test plates were prepared for each antibody type.

Procedure Day 2

- Test plates were incubated for 2 hours at 37°C in a moist chamber.
- Test plates were washed three times in PBS-T using a Biotek automated plate washer.
- 1% skim milk blocking buffer (200 μ l) was added to each well and plates incubated for 1h at 37°C.
- Blocking buffer was emptied from each well. Monoclonal antibodies were diluted in 0.2% skim milk dilution buffer. Anti-A (HE-103, Thermo-Fisher Scientific), Anti-B (89-F, Thermo-Fisher Scientific) and Anti-H (97-I, Thermo-Fisher Scientific) antibodies were diluted 1:2000. Anti-Leb (Seraclone 808423, Bio-Rad) were diluted 1:500. Anti-Lea antibodies (Seraclone 808404, Bio-Rad) were diluted 1: 1000. Diluted primary antibodies (100 μ l) were added to each well of the appropriate test plate and incubated for 1.5 hours at 37°C in a moist chamber.
- Test plates were washed three times in PBS-T using a Biotek automated plate washer.
- Enzyme-conjugated secondary antibody Anti-Mouse IgM – peroxidase antibody produced in goat (Sigma-Aldrich) was diluted 1:5000 in dilution buffer. 100 μ l of diluted secondary antibody was added to each well and incubated for 1.5 hours at 37°C in a moist chamber.
- Test plates were washed three times in PBS-T using a Biotek automated plate washer.
- Enzyme substrate 3',3',5',5'-tetramethylbenzidine, supersensitive, for ELISA (Sigma-Aldrich) (100 μ l) was added to each well and incubated for 10 minutes in darkness.
- The reaction was stopped by 100 μ l of 1M H₂SO₄ to each well.
- Absorbance was read at 450nm using a Biotek microtitre plate reader.

Lectin phenotyping ELISA

Procedure Day 1

Saliva samples were diluted 1:1000 in 0.05M Carbonate Bicarbonate coating buffer (pH 9.6 \pm 0.2). 100 μ l of diluted saliva was added to each well of a 96 well plate (Nunc-Microsorp). Test plates were incubated for 2 hours at 37°C then overnight at 4°C. For blanks and negative controls, 100 μ l of coating buffer was added.

Procedure Day 2

- Test plates were washed three times in PBS-T using a Biotek automated plate washer.
- 1% skim milk blocking buffer (200 μ l) was added to each well and plates incubated for 1h at 37°C.
- Blocking buffer was emptied from each well. Lectin peroxidase conjugate (L8146, Sigma-Aldrich) was diluted 1:3200 in 0.02% skim milk dilution buffer. 100 μ l of diluted conjugate was added to each well and incubated for 1.5h 37°C in a moist chamber.
- Test plates were washed three times in PBS-T using a Biotek automated plate washer.
- Enzyme substrate 3',3',5',5'-tetramethylbenzidine liquid substrate, supersensitive for ELISA (SIGMA-ALDRICH) (100 μ l) was added to each well and incubated for 10 minutes in darkness.
- The reaction was stopped by 100 μ l of 1M H₂SO₄ to each well.
- Absorbance was read at 450nm using a Biotek microtitre plate reader.

Controls and interpretation of results

A positive control sample and negative control sample were included on each plate. In addition, each plate included a "Primary Blank" (no sample, no primary antibody) and "Blank" sample (all except sample). Plates were repeated if the positive control was negative, or negative control positive. The threshold for a positive sample was determined by calculating four times the mean OD of the negative and blank samples. A borderline sample was one with an OD within \pm 0.1 of this threshold.

2.15 HBGA FUT2 GENOTYPING

DNA Extraction

Human DNA extraction from blood for Fut2 genotyping was performed using the Qiagen QIAamp DNA Blood Mini Kit, in accordance with the manufacturers' instructions. The procedure was as follows:

Preparation of Reagents

- Qiagen protease was prepared with the addition of 5.5mL protease solvent.
- Buffer AW1 and AW2 were prepared with the addition of Ethanol (96-100%) in accordance with manufacturer's instructions.

Extraction Spin Protocol

The extraction protocol was as follows:

- All samples and frozen/cooled buffer mixes were allowed to reach room temperature.
- Qiagen protease (20µl) was added to a 1.5mL microcentrifuge tube
- Whole blood sample (200µl) then Buffer AL (200µl) were added to the tube containing protease and mixed by pulse vortexing for 15 seconds.
- The sample solution was incubated at 56°C for 10 min
- Ethanol (96-100%) (200µl), was added to the sample solution, and mixed by pulse vortexing for 15 seconds.
- The sample solution was applied to the spin column (in a 2mL collection tube) then centrifuged at 6000g (8000rpm) for 1 min. The spin column was placed into a clean 2mL collection tube, and the filtrate discarded.
- Buffer AW1 (500µl) was added to the spin column, then centrifuged at 6000g (8000rpm) for 1 min. The spin column was placed into a clean 2mL collection tube, and the filtrate discarded.
- Buffer AW2 (500µl) was added to the spin column, then centrifuged at full speed 20,000g (14,000rpm) for 3 mins.
- The spin column was placed into a clean 2mL collection tube, and the filtrate discarded. The spin column was centrifuged again at full speed for 1 minute.

- The spin column in was placed into a clean 1.5mL microcentrifuge tube and the filtrate discarded. Buffer AE (100μl) was added to the spin column. The spin column was closed and incubated at room temperature for 1 min, then centrifuged at 6000 x g(8000 rpm) for 1 min to obtain eluted DNA. This final step was repeated to obtain eluted RNA.
- Eluted DNA was stored at -20°C

HBGA FUT2 Genotyping: PCR and gel electrophoresis

The FUT2 gene expression determines secretor phenotype. Inactivating mutations of FUT2 result in a non-secretor phenotype. Amplification of the FUT2 gene was performed by PCR (primers detailed in Table 2-16), and restriction fragment length polymorphism used to identify inactivating mutations. The most common mutation, “G428A” affects restriction sites for the enzyme A_{va}II (G’GWCC where W=A or T). A_{va}II was used to cut the PCR product, and fragments examined using gel electrophoresis. Homozygous secretors (two functional copies of FUT2 gene) were identified by one 136 base pair and one 59 base pair fragment. Homozygous non-secretors (two copies of FUT2 gene with inactivating mutation) were identified by unrestricted PCR product (195 base pairs). Heterozygous secretors (one functional copy, one inactivating mutation) were identified by three bands: unrestricted PCR product (195 base pairs), and two fragments (136 and 59 base pairs). Methods were adapted from Marionneau et al. (2005)

PCR amplification of FUT2 gene

Table 2-16: Composition of FUT2 primers and probes

Primer/Probe	Sequence (5’-3’)
FUT2 F	GAGGAATACCGCCACATCCCGGGGAGTAC
FUT2 R	ATGGACCCCTACAAAGGTGCCCCGCCGGCT

FUT2 PCR mastermix was prepared as per Table 2-17. Mastermix (45μl) and 5μl of extracted DNA was then added to each PCR tube. Tubes were briefly centrifuged. Cycling conditions were 95°C for 5minutes, then 30 cycles of 94°C for 1 minute, 45°C for 2 minutes, 72°C for 1 minute. Finally, 72°C for 7 minutes then hold at 15°C.

Table 2-17: Mastermix for FUT2 PCR

Reagents	μl /reaction
10X buffer (Invitrogen)	5.0
50mM MgCl ₂	2.5
dNTPs (10mM)	1.0
Taq polymerase 5U/μl (Invitrogen)	0.3
FUT2-F (10μM)	1.0
FUT2-R (10μM)	1.0
Nuclease free Water	34.2
TOTAL	45.0

Identifying the Avall restriction site

Mastermix for the restriction step was prepared as per Table 2-18. Mastermix (25μl) and 5μl of extracted DNA was then added to each PCR tube. Tubes were briefly centrifuged and incubated at 37°C for 15 minutes then inactivated at 80°C for 20 minutes.

Table 2-18: Mastermix for FUT2 restriction step

Reagents	μl /reaction
10X CutSmart	3.0
Avall	1.0
Nuclease free Water	21
TOTAL	25

Agarose gel electrophoresis

A 2.5% agarose gel was prepared using 2.5g of Ultra-pure agarose (Life Technologies) in 100mL 1X TBE (Tris-borate buffer), with 3.5μl Ethidium Bromide. The cooled gel was poured onto a gel plate until set and transferred to an electrophoresis tank containing 0.5% TBE.

10μl of initial PCR product and 10μl of restricted product were diluted with 10μl of gel loading buffer (Blue/orange loading dye, Promega) and loaded in adjacent slots on the gel. A 100bp DNA ladder (O'Gene) was added for comparison. Electrophoresis was begun at a constant voltage of 120V until sufficient separation was achieved. Gels were then transferred to the UV transilluminator for visualization and identification of DNA fragments.

An illustrative example is given in Figure 2-8.

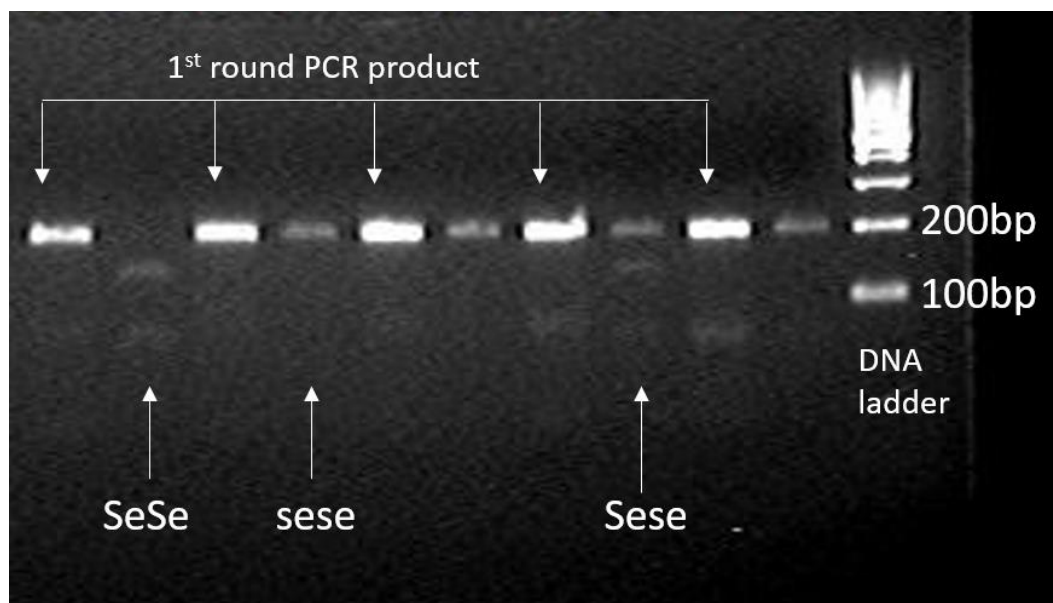


Figure 2-8: Illustrative example of HBGA FUT2 genotyping
First round PCR products are seen at 195bp. SeSe= Homozygous secretor. Two fragments are seen at 59bp and 136 bp. sese=homozygous non-secretor – no restriction by Avall. Only 1 band is seen at 195bp. Sese= heterozygous secretor. Three bands are seen at 195bp, 136bp and 59 bp.

2.16 DATA MANAGEMENT

Study participants were assigned a unique identifier at recruitment. A secure and encrypted database linking identifiable patient data to study identifier was maintained separate to the main study database. All data within the main study database was linked to the unique identifier, with no patient identifiable data. Source data was captured using paper case record forms. Intelligent character recognition scanning software (Cardiff Teleform® Version 10.7, Vista, California) was used to convert data into electronic form. Any queries or discrepancies were manually checked against the study case record form.

2.17 STATISTICAL ANALYSIS

Statistical analysis is described in detail in each chapter. In brief, summary statistics were described as mean and 95% confidence interval for normally distributed continuous variables, median and intra-quartile range for non-normally distributed continuous variables and proportion and 95% confidence interval of proportion for categorical variables. To explore both linear and threshold effects, some variables were analysed as both continuous variables, and

categorized in quantiles to create ordinal variables. Correlations between non-parametric continuous variables were made by Spearman's correlation.

In the cohort study, the relationship between categorical outcomes and predictor variables was evaluated by binomial logistic regression. Multivariable models were created using binomial logistic regression, or by Poisson regression with robust errors where there was failure of the binomial logistic model to converge.

In the case control study, the relationship between categorical outcomes and predictor variables was evaluated by logistic regression for unmatched analysis, or conditional logistic regression for matched analysis. Separate analyses were examined comparing cases to non-rotavirus gastroenteritis controls, and comparing cases to community controls. Multivariable models were created using conditional logistic regression. The Benjamini-Hochberg procedure was applied to adjust for multiple comparisons, with a 25% false discovery rate. (Benjamini and Hochberg, 1995). All analysis was performed in StataIC Version 13.1 (StataCorp, US).

2.18 SAMPLE SIZE

Cohort study sample size

For linear regression analysis, a sample size of 200 was calculated as sufficient (>90% power, $\alpha=0.05$) to detect a minimum linear correlation of ± 0.3 between all primary outcome measures (RV-specific IgA or RV vaccine virus shedding) and continuous predictor variables (passively acquired RV-specific IgG and poliovirus vaccine virus faecal shedding).

For analysis of categorical variables sample size was calculated based on population prevalence of the factors of interest. Seroconversion rate (post-immunisation RV-specific IgA >20IU) was estimated at 50%. RV vaccine shedding rates were estimated at 20-40%. Prevalence of the HBGA phenotypes of interest (Lewis negative, non-secreter) were estimated at 20-25%.

Based on these assumptions a sample size of 200 would achieve:

- Detection of a reduction in the seroconversion rate from 50% in those with virus shedding to 25% in those infants without shedding (assuming a minimum population shedding rate of 20%) with 81% power.
- Detection of a reduction in the seroconversion rate from 50% in Lewis positive/secretor infants to 25% in Lewis negative/non-secretor infants (assuming a minimum Lewis negative/non-secretor prevalence of 25%) with 85% power.
- Detection of a reduction in shedding rate from 30% in Lewis positive/secretor infants to 9.9% in Lewis negative/non-secretor infants (assuming a minimum Lewis negative/non-secretor prevalence of 25%) with 81% power.

Case:control sample size

Sample size was based on odds ratio of vaccine failure by HBGA phenotype and was calculated based on the estimated proportion of the phenotypes of interest (Lewis negative or non-secretors) in controls. Table 2-19 shows sample size requirements by varying HBGA prevalence (Dupont, 1988). Assuming a minimum population prevalence of HBGA phenotype of interest of 20%, a sample size of 123 cases (plus 123 community and 123 non-rotavirus gastroenteritis controls) achieves 80% power to detect an odds ratio of 2.5 versus the alternative hypothesis of equal odds using a Chi-Square test with a 0.05 alpha significance level.

Table 2-19: Sample size estimates for Case:Control study

% Non-secretor/ Lewis negative phenotype*	OR 2.0	OR 2.5	OR 3.0
20	222 (444)	123 (246)	84 (168)
25	196 (392)	110 (220)	76 (152)
30	180 (360)	102 (204)	71 (142)
40	167 (334)	97 (194)	68 (136)

* In community controls. Figure in bold= number of cases required. Figure in brackets=total number with controls required. Sample size calculated for 1:1 matched case:control, power 0.8, α 0.05. Correlation coefficient is estimated at 0.2. Shaded area = 0.8 power for sample size of 125 cases.

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3 PREDICTORS OF VACCINE VIRUS REPLICATION

3.1 INTRODUCTION

Understanding of exact pathways of rotavirus infection and immunity in humans remains elusive. Rotavirus attaches to the intestinal mucosa, gains entry to enterocytes, sequesters host cell machinery to replicate the viral genome and repackage new virions before release and repeat. Infection of the host cell triggers an innate immune response, including induction of immunomodulatory and anti-viral proteins, and priming of the adaptive immune response (Arnold et al., 2013). For longer term protection, humoral immunity appears key, mediated by RV-specific B cells (Clarke and Desselberger, 2015).

Effective live oral vaccines mimic natural infection to stimulate a mucosal immune response and generate adaptive immunity against future disease. Live oral vaccines must strike a careful balance: attenuated enough to be safe, but virulent enough to ensure adequate viral replication to generate a robust adaptive immune response. Rotavirus vaccine virus replication in the host gut is therefore an essential step toward generating protective immunity, and an important measure of vaccine “take”.

Direct measurement of vaccine viral replication in the gut mucosa is clearly not feasible *in vivo*. Vaccine virus shedding in stool is therefore used as a proxy measure. Initial studies reporting rotavirus vaccine virus shedding used rotavirus antigen detection, however this method proved insufficiently sensitive. The development of molecular methods of rotavirus detection, particularly RV1 vaccine-specific NSP2 RT-PCR, offers opportunities to more accurately estimate vaccine virus shedding, and by proxy, vaccine take. Since inhibition of vaccine virus replication is the mechanism of several proposed causes of lower rotavirus vaccine efficacy in low-income settings (Figure 3-1), data on vaccine virus shedding could be highly informative. Current evidence for each proposed cause of reduced rotavirus vaccine efficacy is summarized in detail in Chapter 1, however the suggested mechanism of each will be described briefly.

Rotavirus-specific IgG, passively acquired through transplacental transfer, may contribute to protection against rotavirus infection in early life. The natural history of rotavirus infection, with

symptomatic infection in early infancy rare and peak age of infection around the time of waning maternal immunity around 5-8 months of age, does support the concept of some degree of passively-acquired immunity (Steele et al., 2016). However, mechanisms of IgG mediated immunity are not well understood, and studies on the protective effect of RV-specific IgG have produced variable results. In principle, high RV-specific IgG could reduce vaccine virus replication in the same way it protects against natural infection (Ray et al., 2007). Maternal RV-specific IgG is likely to be higher in high-burden, low-income countries like Malawi.

Like RV1, OPV is a live, oral virus vaccine. Concurrent administration could reduce RV1 vaccine virus replication by competitive inhibition at any step of the replication pathway. OPV is a trivalent vaccine, comprising three Sabin sub-types. Sabin 2 has been shown to inhibit shedding of the other two sub-types, providing proof of principle of direct vaccine virus competition. Wang et al. (2012) in an *in-vitro* study of co-infection of cells by rotavirus alone or in combination with enteroviruses, showed suppression of rotavirus infectivity in co-infection.

Histo-blood group antigens (HBGAs) have been proposed as genotype specific receptors for rotavirus cell attachment, mediating cell entry and viral replication. In support of this, HBGA phenotype has been associated with genotype-specific susceptibility to rotavirus infection, with non-secretor (and possibly Lewis negative) individuals apparently resistant to P[8] rotavirus infection. Resistance to P[8] infection could extend to the G1P[8] based monovalent vaccine, resulting in reduced vaccine virus replication.

Finally, a complex combination of enteric infection, dysbiosis of the gut microbiota and environmental enteropathy could contribute to an infant gut unfavourable to rotavirus replication. Direct assessment of co-infection, dysbiosis and enteropathy is beyond the scope of this thesis. However, in an exploratory analysis I examined the effect on vaccine virus replication of potential causative factors of poor gut health: poverty, poor sanitation, low birth weight, HIV exposure and malnutrition.

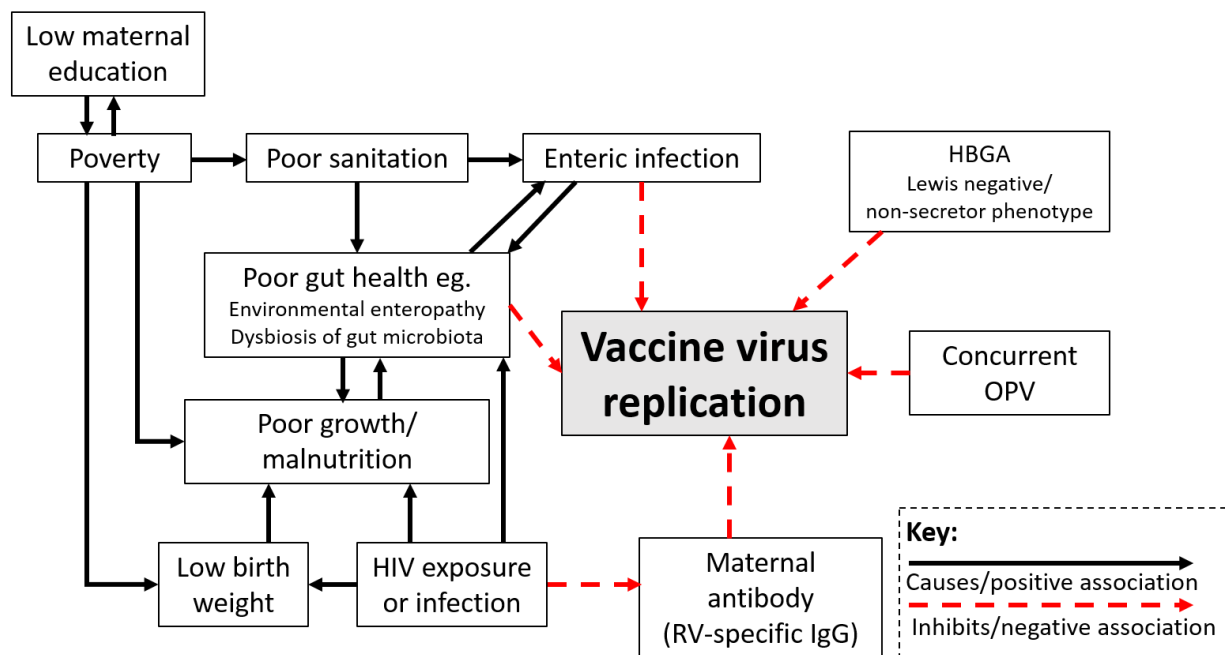


Figure 3-1: Factors which may inhibit rotavirus vaccine virus replication

3.2 AIMS

As RV1 vaccine virus shedding has not previously been assessed in this population, the first aim of the study was to describe the pattern of post-RV1 vaccine virus faecal shedding in Malawian infants. The study then aimed to determine the relationship between passively-acquired maternal antibody, oral polio vaccine response, HBGA phenotype and rotavirus vaccine virus shedding and explore the following hypotheses:

3.2.1 Hypotheses

Primary hypotheses

1. Passively-acquired maternal antibody (RV-specific IgG)

Higher levels of passively-acquired maternal rotavirus antibody (RV-specific IgG) at time of 1st RV1 immunisation will be associated with lower vaccine virus shedding.

2. Oral polio vaccine response

OPV shedding at time of rotavirus immunisation will be associated with lower rotavirus vaccine virus shedding.

3. HBGA phenotype

- a. Lewis negative HBGA phenotype will be associated with lower vaccine virus shedding.

- b. Non-secretor HBGA phenotype will be associated with lower vaccine virus shedding.

Secondary Hypotheses

4. Other host factors

- a. LBW infants will have lower vaccine virus shedding.
- b. HIV exposed infants will have lower vaccine virus shedding.
- c. Infants with poor nutritional status will have lower vaccine virus shedding.

5. Socioeconomic and Sanitation factors

- a. Predictors of poverty will be associated with lower vaccine virus shedding.
- b. Predictors of poor sanitation will be associated with lower vaccine virus shedding.

3.3 METHODS

3.3.1 Recruitment and data collection

Detailed methods are given in Chapter 2. In brief, this was a longitudinal cohort study. Infants were recruited from Zingwangwa Health Centre, Blantyre, Malawi, prior to 1st RV1 immunisation at around 6 weeks of age. Infants with known conditions causing immunosuppression (other than HIV) and those not expected to be resident in Blantyre throughout the study period were excluded. Wild type rotavirus infection at time of immunisation (as determined by VP6 qRT-PCR) was not an exclusion criteria.

Social and demographic data were collected by structured interview. Infant characteristics, including low birth-weight and gender, and key socio-economic data which could be associated with reduced vaccine virus shedding were recorded. Definitions are provided in Chapter 2.6.

Infant and maternal HIV status was determined by verbal report and from hand-held health records as detailed in Chapter 2.6. HIV-exposure was defined as any infant of an HIV-infected mother. HIV-infection in infants was confirmed by DNA PCR test at 6 weeks. HIV DNA PCR tests were performed by Ministry of Health clinical staff and not as part of the study. Results were reported by families to the study team when available.

Nutritional status was determined by length, weight and mid-upper arm circumference measurement at time of recruitment, and at each follow-up visit, and compared to WHO age-determined z scores.

3.3.2 Laboratory methods

Laboratory methods are described in detail in Chapter 2.

The primary outcome measure was RV1 vaccine virus shedding. Stool samples for RV1 shedding were taken prior to each RV1 dose and on alternate days from days 2-10 post immunisation. A minimum of two post-immunisation samples were required for infants to be included in the shedding analysis. RV1 shedding was determined by vaccine-specific NSP2 RT-PCR and confirmed by VP6 qRT-PCR. RV1 vaccine virus shedding was defined as two or more positive NSP2 positive, VP6 positive samples from days 4-10 post immunisation. Level of shedding was quantified using peak shedding, defined as the reciprocal of the minimum NSP2 Ct value of all positive samples within each dose period.

The primary predictive measures were RV-specific IgG, OPV virus shedding, poliovirus-specific neutralizing antibody response and HBGA phenotype.

RV-specific IgG at baseline was determined by sandwich ELISA.

OPV virus shedding was determined by multiplex RT-PCR for Sabin sub-types 1-3. OPV virus shedding was defined as any two positive samples of any Sabin sub-type from days 4-10 post immunisation. The relationship between RV1 shedding and OPV shedding was determined with OPV shedding as a categorical variable, both overall and by predominant Sabin sub-type. The predominant Sabin sub-type shedding pattern for each infant was defined as the most frequently occurring Sabin sub-type combination in each dose period. Where no single combination was most frequent, the combination with the most Sabin sub-types was selected. Predominant Sabin sub-types were defined as the most frequently occurring sub-type, alone or in combination, within a dose period. Peak shedding for each Sabin sub-type was determined by the reciprocal of the minimum Ct value of all positive samples within a dose period.

Poliovirus-specific neutralizing antibody responses to serotypes 1 and 3 were determined prior to routine immunisation around 6 weeks, and two weeks following the subsequent dose. Poliovirus-specific neutralizing antibody responses to serotype 2 could not be tested due to the ban on use of live Sabin 2 viruses in laboratories following the global switch to bivalent OPV in April 2016. Neutralizing antibody titres were determined using a micro-neutralization assay. Titres greater than 8 were considered protective (seropositive)(Sutter et al., 1995). This definition and methodology used are consistent with WHO agreed international standards for polio serology. Poliovirus-specific seroconversion between six and twelve weeks was defined as either a change from seronegative at six weeks to seropositive at twelve weeks, or greater than a four-fold rise in titres between six and twelve weeks.

HBGA phenotype at baseline was determined by salivary ELISA for A, B, H and Lewis a and b antigens. Infants who were A, B or H positive were classified as secretors. Infants who were negative for ABH were confirmed as non-secretors by Lectin ELISA and by FUT2 genotyping. Infants with saliva positive for either Lewis a or b antigen were classed as Lewis positive, and those negative for both Lewis antigens as Lewis negative. Final secretor and Lewis phenotype was determined by two ELISA tests (ABH or Lectin) confirming the same phenotype. HBGA phenotype at time of 2nd dose was determined only for infants who were non-secretors at 1st dose. The relationship between RV1 shedding after each dose and HBGA phenotype was based on HBGA phenotype determined at that specific dose. The relationship between overall RV1 shedding and HBGA phenotype was based on HBGA phenotype at baseline, but with sensitivity analyses based on HBGA phenotype at 2nd dose and HBGA genotype.

3.3.3 Statistical analysis

Summary statistics were described as mean and 95% confidence interval for normally distributed continuous variables, median and inter-quartile range for non-normally distributed continuous variables and proportion and 95% confidence interval of proportion for categorical variables. To explore both linear and non-linear effects, IgG and shedding variables were analysed as both continuous variables, and categorized in quantiles to create ordinal variables. Correlations between non-parametric continuous variables were made by Spearman's

correlation. Relationship between categorical outcomes and predictor variables was made by binomial logistic regression. Multivariable models were created using binomial logistic regression, or by Poisson regression with robust errors where there was failure to converge. All variables significant to $p < 0.10$ in univariable analysis were included in the multivariable model. The model was built using a manual stepwise backward elimination approach, whereby variables with the highest p value were removed sequentially until only variables with $p < 0.1$ remained. The Benjamini-Hochberg procedure was applied to adjust for multiple comparisons, with a 25% false discovery rate (Benjamini and Hochberg, 1995). Statistical analysis was performed in StataIC version 13.1 (StataCorp, US).

3.4 RESULTS

3.4.1 Recruitment

Recruitment screening for the cohort study began in April 2015 with the first participant commencing the study in May 2015. Recruitment was completed in August 2016.

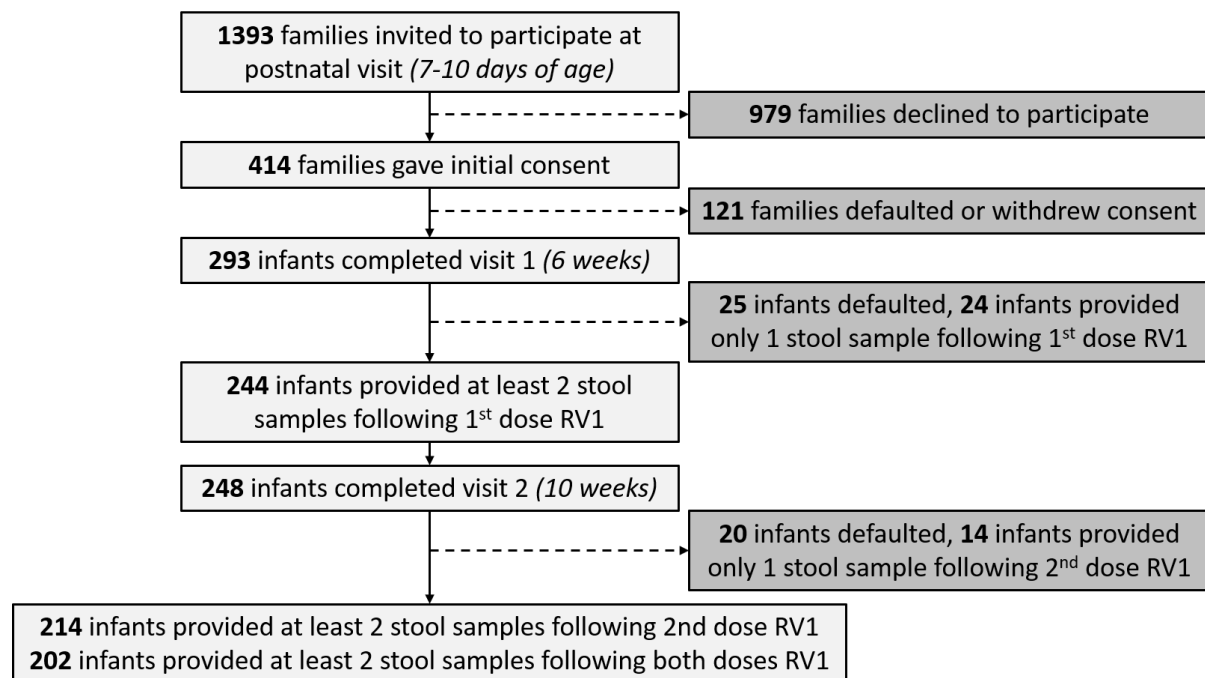


Figure 3-2: Recruitment Flow Diagram for Shedding Cohort

Recruitment for the cohort study is shown in Figure 3-2. Attrition was 29% between initial consent in the postnatal period and completion of the first study visit at 6 weeks. From recruitment at 6 weeks to completion of the study attrition was 21%. This attrition was primarily due to withdrawal of consent following consultation with the wider family, or due to participants leaving the area to attend the harvest or for other family reasons. Infants who remained in the study were demographically similar to those who withdrew (see 3.4.2) and also to the infants recruited as community controls in the case:control study (Chapter 5). Despite attrition, it is therefore reasonable to assume that the study population is locally representative.

3.4.2 Demographics of shedding cohort

Demographics of infants with complete shedding data are summarized in

Table 3-1. Vaccination was highly timely, within 1 week of the scheduled dates. Prevalence of low-birth weight and HIV exposure were as expected for this setting (see Chapter 2.6). Of 28 HIV exposed infants, 11 had an HIV DNA PCR result known by the end of follow-up – all were HIV uninfected. All HIV exposed infants had received anti-retroviral prophylaxis. Measures of nutritional status at 6 weeks were within expected WHO range for age. There was a high proportion of families with indicators of poor sanitation, poverty and food insecurity as expected for this low-income peri-urban setting (see Chapter 2.6). Almost all infants were breast-fed.

Table 3-1: Demographics of Shedding Cohort

Characteristic	Complete Shedding Cohort (n=202)
<u>Timing of vaccine</u>	
Median age in weeks at 1 st RV1 dose (IQR)	6.1 (6.0-6.3)
Median age in weeks at 2 nd RV1 dose (IQR)	10.6 (10.4-10.9)
Median time between RV1 doses, weeks (IQR)	4.4 (4.3-4.4)
<u>Infant characteristics</u>	
Male	101 (50%, 43-57%)
Exclusively breast-fed	201 (99%, 97-100%)
HIV-exposed	28/201 (14%, 10-19%)
Low birth weight (<2.5kg)	21/197 (11%, 7-16%)
<u>Nutritional status</u>	
Median weight for age z-score at 1 st RV1 dose (IQR)	-0.38 (-1.04-0.26)
Median length for age z-score at 1 st RV1 dose (IQR)	-0.69 (-1.83- -0.03) (n=199)
Median weight for length z-score at 1 st RV1 dose (IQR)	0.5 (-0.45-1.33)
Median MUAC at 1 st RV1 dose, cm (IQR)	12.5 (11.6-13)
<u>Sanitation and socioeconomic predictors</u>	
Median household size (IQR)	5 (4-6)
Non-piped water source (borehole or well)	22 (11%, 8-16%)
Time taken to access water	
<5minutes	83/201 (37%, 31-44%)
5-30 minutes	106/201 (54%, 46-60%)
>30 minutes	20/201 (10%, 6-15%)
Pit-latrine type toilet	180/199 (91%, 85-94%)
Electricity at home	105 (52%, 45-59%)
One or more household member with salary	163 (81%, 75-86%)
Household food insecurity	62 (31%, 25-37%)
Median age of head of household, years (IQR)	32 (27-38)
Median years of maternal education (IQR)	10 (8-12)

Categorical variables are reported as number, (proportion, 95%CI of proportion). Denominator n=202 unless otherwise stated. MUAC -mid-upper arm circumference.

Demographic characteristics of infants who completed the study are compared to those who withdrew or defaulted (Table 3-2). Household size was slightly smaller, and head of household slightly younger in those families who did not complete the study. There were no other significant differences in infant or household characteristics demonstrated.

Table 3-2: Demographics of cohort infants, compared to those who withdrew or defaulted

Characteristic	Completed study n=232	Withdrew/Defaulted n=61	p
<u>Infant characteristics</u>			
Male	112 (48%, 42-55%)	34 (56%, 43-68%)	0.30 ^b
Exclusively breastfed			
HIV-exposed	32 (14%, 10-19%)	6 (10%, 5-21%) (n=60)	0.43 ^b
Low birth weight (<2.5kg)	25 (11%, 7-16%)	5 (8%, 3-19%)	0.56 ^b
<u>Nutritional status</u>			
Median weight for age z-score at 1 st RV1 dose (IQR)	-0.39 (-1.04-0.26) (n=232)	-0.26 (-1.04-0.41)	0.40 ^a
Median length for age z-score at 1 st RV1 dose (IQR)	-0.67 (-1.64-0.02) (n=226)	-0.31 (-1.43-0.39)	0.11 ^a
Median weight for length z-score at 1 st RV1 dose (IQR)	0.39 (-0.53-1.33) (n=226)	0.18 (-0.59-1.2)	0.62 ^a
<u>Sanitation and socioeconomic predictors</u>			
Median household size (IQR)	5 (4-6)	4 (3-5)	0.02^a *
Non-piped water source	27 (12%, 8-16%)	9 (15%, 8-26%)	0.50 ^b
Time taken to access water (n=231)			
<5minutes	83 (36%, 30-42%)	26 (38%, 31-56%)	0.39 ^c
5-30 minutes	125 (54%, 48-61%)	32 (53%, 40-65%)	
>30 minutes	23 (10%, 7-15%)	3 (10%, 2-15%)	
Pit-latrine type toilet	207/229 (90%, 86-94%)	52/57 (91%, 80-96%)	0.54 ^c
Electricity at home	121 (52%, 46-59%)	34 (56%, 43-68%)	0.62 ^b
One or more household member with salary	184 (79%, 74-84%)	48 (79%, 66-87%)	0.92 ^b
Household food insecurity	70 (30%, 25-36%)	16 (26%, 17-39%)	0.55 ^b
Median age of head of household (IQR)	32 (28-38)	28 (25-36)	0.04^a *
Median years of maternal education (IQR)	10 (8-12)	10 (8-12)	0.94 ^a

Categorical variables are reported as number, (proportion, 95%CI of proportion). Denominator n=232 (completed cohort, includes infants with immunogenicity but insufficient data for shedding analysis) or n=61 (withdrew/defaulted) unless otherwise stated. a. Kruskal-Wallis equality of populations rank test. b. Chi² test c. Fisher's exact test *significant difference at p<0.05 level.

3.4.3 Rotavirus vaccine virus faecal shedding following RV1 immunisation

With shedding defined as two or more positive samples within the post-dose period, 73/244 (30%, 95%CI 25-36%) infants shed vaccine virus after the 1st dose RV1, 70/214 (33%, 27-39%) after the 2nd dose RV1 and 104/202(52%, 45-58%) after either vaccine dose.

Missing stool samples were common, due to logistical challenges and irregular stooling patterns in breast-fed infants: 96/244 (39%) infants were missing at least 1 stool sample after 1st dose RV1, 77/214(36%) after 2nd dose RV1 and 116/202(57%) infants were missing at least 1 stool sample after at least 1 dose. Missing samples appeared to be missing at random.

In a sensitivity analysis, there was no significant difference in the distribution of shedding after either dose when infants with missing samples were compared to those with no missing samples (Table 3-3).

Table 3-3 RV1 shedding: complete data versus incomplete data

	No missing stool samples	At least 1 missing stool sample	Chi² p value
Shedding after 1 st dose RV1	29% (22-37%)	31% (23-41%)	0.71
Shedding after 2 nd dose RV1	35% (27-44%)	29% (19-40%)	0.33
Shedding after either dose RV1	52%(42-63%)	51%(42-60%)	0.84

Individual shedding patterns for 202 infants with sufficient data available for both dosing periods are shown in Figure 3-4. Four main shedding patterns emerged. 98/202(49%, 42-55%) infants did not meet shedding criteria (had fewer than two positive samples) after either RV1 dose. 37/202 (18%, 95%CI 14-24%) infants shed vaccine virus after dose 1 only. 47/202 (23%, 95%CI 18-30%) infants shed vaccine virus after dose 2 only. 20/202 (10%, 95%CI 6-15%) shed vaccine virus following both doses of RV1. Shedding after dose 1 did not significantly predict shedding after dose 2 (RR 1.1 (0.69-1.7), p=0.72).

Overall, RV1 shedding was low level, with a median Ct value of 29.9 (IQR 26.5-32.7) following the 1st dose and 32.7 (IQR 30.7-34.9) following the 2nd dose.

Peak shedding, defined as the reciprocal of the minimum Ct value, was significantly higher in infants with shedding after 1st dose RV1 only, than in infants with shedding after 2nd dose RV1 only (Figure 3-3). In infants with shedding after both doses, peak shedding for each post-dose period was similar to infants with shedding only after dose 1 and dose 2 respectively.

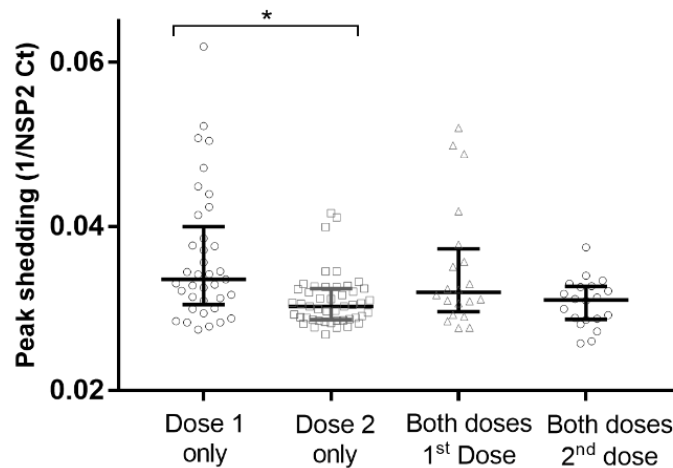


Figure 3-3: Peak vaccine virus shedding by shedding pattern

Peak vaccine virus shedding (1/minimum Ct value) is shown by shedding pattern. Dose 1 only: infants with RV1 shedding after the 1st dose only (median Ct 29.8 (IQR 25.9-32.3)); "Dose 2 only"- infants with RV1 shedding after the 2nd dose only (median Ct 33.1 (IQR 30.8-37.2)); and "Both doses" – infants with RV1 shedding following both doses (median Ct 31.3 (IQR 27.2-33.4) after dose 1 and 32.2 (IQR 30.6-34.9) after dose 2). *Wilcoxon rank sum test $p < 0.001$. Error bars show median with inter-quartile range.

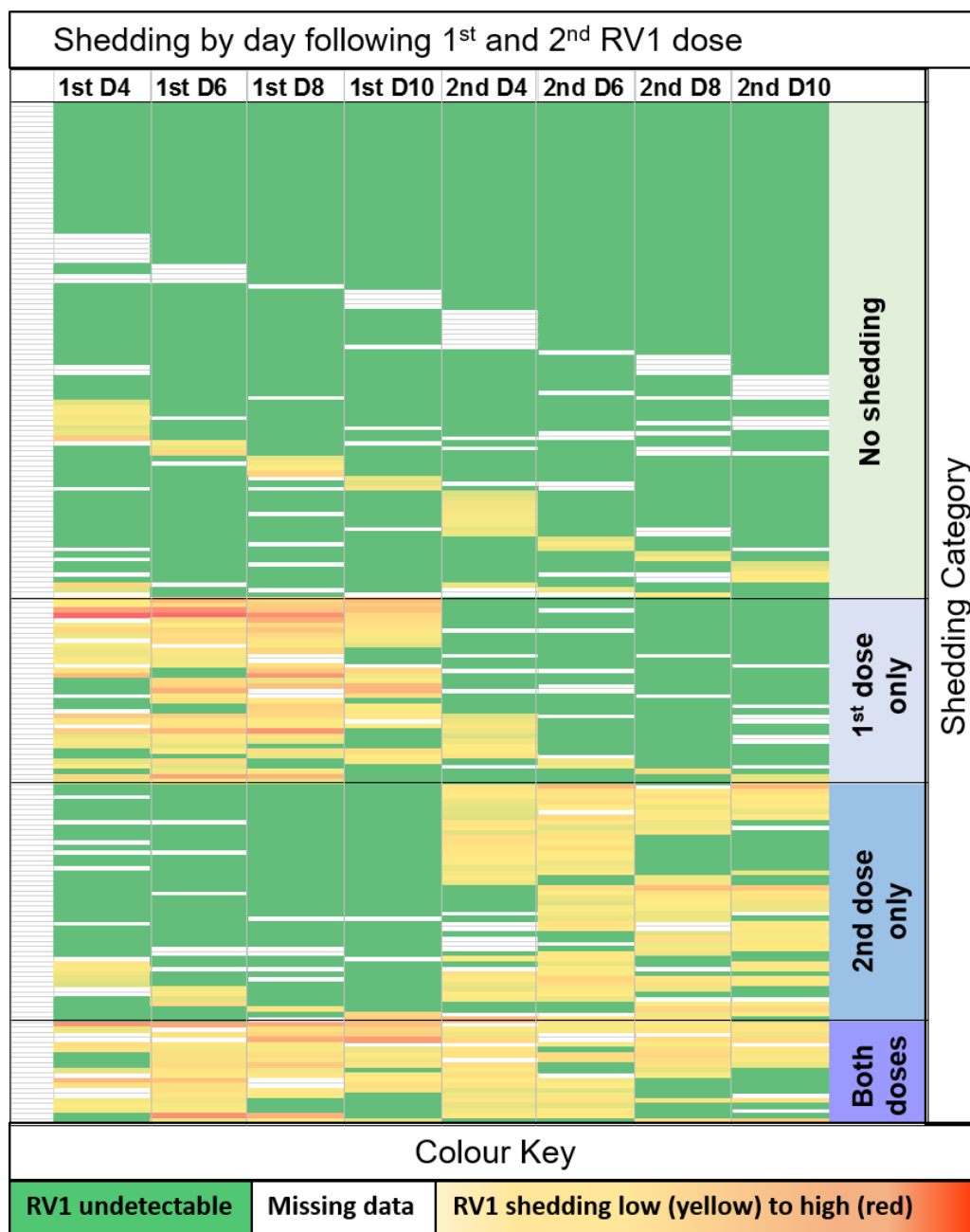


Figure 3-4: RV1 vaccine virus shedding patterns

Clustered heatmap showing RV1 vaccine virus shedding patterns. Shedding was defined as 2 or more positive samples following either dose. Infants are grouped into four shedding categories: “No shedding” – infants who did not meet shedding criteria; “1st dose only”- infants with detectable RV1 shedding after the 1st dose only; “2nd dose only”- infants with detectable RV1 shedding after the 2nd dose only; and “Both doses” – infants with detectable RV1 shedding following both doses. Columns show shedding on each alternate day from day 4-10 following 1st and 2nd RV1 doses. Each row shows data for one infant (n=202). Colours indicate the level of RV1 shedding (determined by semi-quantitative RT-PR) Undetectable shedding is shaded green. Low shedding to high shedding is shaded on a gradient from yellow to red. Missing data, where a stool sample was not provided, is shown in white.

A positive sample on day 6 best predicted likelihood of rotavirus shedding after both doses one and two, and increased the overall likelihood of RV1 shedding after either dose three-fold (Table 3-4). A positive sample on either day 6 post dose 1, or day 6 post dose 2, predicted overall RV1 shedding with a sensitivity of 85.4% and specificity of 92.9% (RR 6.5 (95%CI 4.1-10.5), $p < 0.001$).

Table 3-4: Prediction of overall shedding by sample day

Shedding on sample day	RV1 shedding after 1st dose RR (95%CI)	p	RV1 shedding after either dose RR (95%CI)	p
1 st dose D2	1.9 (1.2-3.2)	<0.01	1.4 (1.0-1.8)	0.03
1 st dose D4	6.2 (3.6-10.8)	<0.001	2.0 (1.6-2.7)	<0.001
1 st dose D6	22.8 (9.6-54.0)	<0.001	2.9 (2.3-3.8)	<0.001
1 st dose D8	19.8 (9.0-43.5)	<0.001	2.6 (2.1-3.4)	<0.001
1 st dose D10	7.2 (4.6-11.2)	<0.001	2.2 (1.8-2.8)	<0.001
Shedding on sample day	RR of RV1 shedding after 2nd dose RR (95%CI)	p	RV1 shedding after either dose RR (95%CI)	p
2 nd dose D2	2.1 (1.3-3.4)	0.002	1.6 (1.2-2.1)	0.003
2 nd dose D4	6.5 (3.7-11.4)	<0.001	2.6 (2.0-3.5)	<0.001
2 nd dose D6	10.1 (5.7-18.0)	<0.001	3.0 (2.3-3.9)	<0.001
2 nd dose D8	6.5 (4.2-9.9)	<0.001	2.4 (1.9-3.0)	<0.001
2 nd dose D10	4.7 (3.2-6.8)	<0.001	2.3 (1.9-3.0)	<0.001

Vaccine virus shedding: sensitivity analyses

The chosen definition of shedding required serial shedding (two or more positive samples within each post-dose period). This resulted in a number of infants with a single positive sample categorized as “no shedding” for that post-dose period. 39/244 (16%) infants following 1st dose RV1 and 36/214 (17%) infants following dose 2 had a single positive sample for vaccine virus. 2/202(1%) infants had a single positive sample after both doses. 39/202(19%) infants had a

single positive sample after either RV1 dose. Single positive samples tended to be low level; peak shedding was significantly lower (1st dose, median 35.8 (IQR 33.4-37.7), 2nd dose, median 36.9 (IQR 34.8-37.9)) for infants with single positive samples than for infants with two or more positive samples (1st dose, median 29.9 (IQR 26.5-32.4), 2nd dose median 32.9 (IQR 30.7-34.9)), Wilcoxon rank sum $p < 0.0001$ for both post-dose periods.

Using a less conservative definition of shedding defined as 1 or more positive samples within each post-dose period, 114/244 (47%, 41-53%) infants shed vaccine virus following dose 1, 108/214 (51%, 44-57%) infants shed following dose 2, and 143/202 (71%, 64-77%) infants shed following either dose.

Using a cut-off of peak shedding cycle threshold of 27 as a proxy for EIA positivity (Bennett et al., 2015) estimated vaccine virus shedding rates by EIA were much lower – only 22/202 (11%, 7-16%) after either dose.

3.4.4 Wild-type rotavirus infection and diarrhoea around time of vaccination

A total of 46/155 (30%, 23-37%) infants with pre-immunisation stool samples at 6 weeks were positive by RT-PCR for wild-type rotavirus infection. Only two of these infants were reported to have current or recent diarrhoea. Only one child in the total cohort of 293 reported diarrhoea requiring healthcare review/treatment in the first 6 weeks of life.

A total of 25/191 (13%, 9-19%) infants with pre-immunisation stool samples at 10 weeks were positive for wild-type rotavirus infection. None of these infants were reported to have current or recent diarrhoea. In addition, 17/191 (9%, 6-14%) infants were shedding RV1 vaccine virus prior to the second RV1 dose.

In total, only seven infants during the 1st vaccine period and seven infants during the 2nd vaccine period were reported to have current or recent diarrhoea.

3.4.5 Passively-acquired maternal antibody and RV1 vaccine virus shedding

Geometric mean concentration of RV specific IgG at 6 weeks was 3621.8 (95% CI 3145.2-4170.6) U/mL. Higher RV-specific IgG was associated with reduced likelihood of shedding after 1st dose RV1: for every 1 log increase in RV-specific IgG, likelihood of RV1 shedding reduced by 23% (Table 3-5). However, this association was not seen for shedding after the 2nd dose.

Comparing the quantitative relationship between RV-specific IgG and peak RV1 vaccine virus shedding, there was no significant correlation between the two following the 1st dose (Spearman's ρ -0.15, $p=0.25$), and only a weak negative correlation following the 2nd dose (Spearman's ρ -0.29, $p=0.02$).

To explore non-linear threshold effects, infants were categorized by RV-specific IgG quartile at 6 weeks. The relationship between shedding and IgG quartile was determined by binomial regression, with RV-specific IgG 1st quartile as the reference category. Infants with RV-specific IgG in the highest quartile were less likely to shed RV1 following the 1st dose, compared to infants with RV-specific IgG in the lowest quartile. A similar, non-significant trend was seen following the 2nd dose (Table 3-5).

Table 3-5: Relationship between RV-specific IgG and RV1 shedding

	Shedding after 1st dose RV1 RR (95% CI)	Shedding after 2nd dose RV1 RR (95% CI)	Shedding after either dose RV1 RR (95% CI)
RV-specific IgG			
1 st quartile	ref	ref	ref
2 nd quartile	0.72 (0.43-1.2) $p=0.23$	0.49 (0.26-0.95) $p=0.03$	0.75 (0.52-1.1) $p=0.14$
3 rd quartile	0.79 (0.48-1.3) $p=0.35$	1.1 (0.71-1.8) $p=0.63$	1.1 (0.79-1.4) $p=0.71$
4 th quartile	0.31 (0.14-0.66) $p=0.002$	0.59 (0.33-1.1) $p=0.08$	0.44 (0.27-0.72) $p=0.001$
Log RV-specific IgG	0.77(0.63-0.93) $p=0.007$	0.87 (0.72-1.0) $p=0.13$	0.83 (0.74-0.95) $p=0.005$

3.4.6 Oral polio vaccine response and RV1 vaccine virus shedding

3.4.6.1 *OPV vaccine shedding patterns and neutralizing antibody response.*

Analysis of the relationship between OPV and RV1 vaccine virus shedding was complicated by the global switch from bivalent to trivalent OPV which was completed in Malawi at the end of April 2016. To avoid confounding from the differing interactions between trivalent and bivalent OPV, only infants who received trivalent OPV (from birth onwards) were included for analysis. This reduced the sample size for OPV analysis from 244 to 171.

In the Malawi immunisation schedule, a birth dose of OPV is encouraged but not repeated if missed. In this cohort, 150/171 (88%, 82-92%) infants received a dose of OPV around the time of birth. In infants who had received a birth dose of OPV with pre-immunisation stool samples available at 6 weeks, 42/85 (49%, 39-60%) were positive for at least one Sabin virus, suggesting prolonged OPV shedding from the birth dose. Of these infants, 26/42 (62%, 46-76%) were shedding Sabin 2 only, 11/42 (26%, 15-42%) Sabin 3 only, 2/42 (5%, 1-18%) Sabin 1 only and 3/42 (7%, 2-21%) a combination of Sabin 1 and 2.

OPV and RV1 are scheduled on the same day. In the study population, only 7/293(2%) infants at 1st RV1 dose and 5/246(2%) infants at 2nd RV1 dose did not receive concurrent OPV, all due to vaccine stock-outs. These infants were excluded from OPV analysis.

OPV shedding was defined as two or more samples positive for at least one Sabin virus within the post-dose period. OPV shedding rates were much higher than for RV1: 142/171 (83%, 77-88%) infants shed OPV in the 1st dose period, 84/132 (64%, 55-71%) shed OPV after the 2nd dose and 111/117 (95%, 89-98%) infants shed after either dose. Of the six infants who did not meet OPV shedding criteria, only one showed no evidence of shedding at all, five had at least one positive sample pre- or post-immunisation.

OPV shedding patterns were complex, with variable shedding of different combinations of the three Sabin sub-types at different time points (Figure 3-5).

Table 3-6: Predominant Sabin sub-type shedding pattern

Predominant Sabin sub-type shedding pattern	1st dose period n=142	2nd dose period n=84
Sabin 1 only	8 (6%, 3-11%)	7 (8%, 4-17%)
Sabin 2 only	30 (21%, 15-29%)	10 (12%, 6-21%)
Sabin 3 only	36 (25%, 19-33%)	31 (37%, 27-48%)
Sabin 1 & Sabin 2	17 (12%, 8-19%)	3 (4%, 1-11%)
Sabin 1 & Sabin 3	22 (15%, 10-23%)	17 (20%, 13-30%)
Sabin 2 & Sabin 3	13 (9%, 5-15%)	5 (6%, 2-14%)
All 3 Sabin types	16 (11%, 7-18%)	11 (13%, 7-22%)

Sabin 3 was the most common predominant sub-type, alone or in combination, across both dosing periods (Table 3-6). Sabin 2 was the most common pre-dose and in the 1st dose period, but was much less commonly found by the 2nd dose period. Despite both occurring commonly, Sabin 2 and 3 were infrequently found in combination together. Sabin 1 was most often found in combination with other sub-types and was rarely found alone.

Overall shedding patterns differed from RV1. Infants most commonly shed RV1 either after the 1st dose, or after the second, but less commonly after both doses. In contrast, 61/117 (52%, 43-61%) infants shed OPV after both doses, 38/117 (33%, 25-42%) only shed OPV in the 1st dose period, and 12/117 (10%, 6-17%) shed OPV only in the 2nd period. In most (48/61, 79%, 66-87%) infants who shed OPV after both doses, the predominant Sabin sub-type combination in the 1st dose period differed from that in the 2nd dose period.

There was some evidence of different distribution of predominant sub-types between different shedding patterns. For example, Sabin 2 was the predominant sub-type in the 1st dose period for 19/61 (31%, 21-44%) of infants who shed after both doses, whereas Sabin 3 was more common (12/38, 32%, 18-49%) and Sabin 2 less common (11%, 4-26%) in infants who shed OPV in the first dose period only. However, numbers were too small to confirm this.

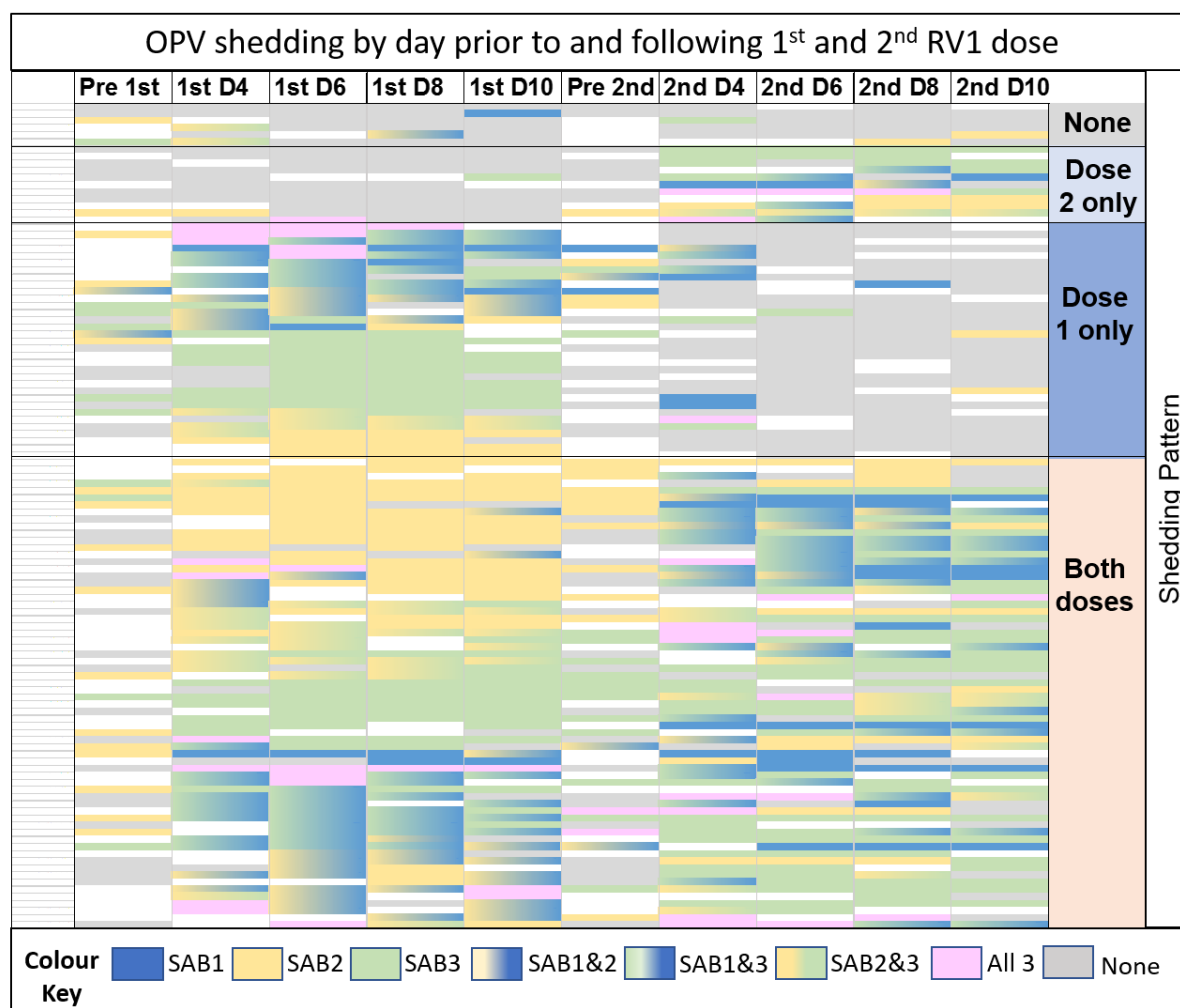


Figure 3-5: OPV shedding patterns

Clustered heatmap showing OPV virus shedding patterns. OPV shedding was defined as 2 or more positive samples of any Sabin sub-type following either dose. Pre-dose samples were not included in the shedding definition. Infants with only 1 positive sample within a post-dose period were categorized as “no shedding”. Infants are grouped into four shedding categories: “No shedding” – infants who did not meet shedding criteria; “1st dose only”- infants with detectable OPV shedding after the 1st dose only; “2nd dose only”- infants with detectable OPV shedding after the 2nd dose only; and “Both doses” – infants with OPV shedding following both doses. Columns show shedding on pre-dose and each alternate day from day 4-10 following 1st and 2nd RV1 doses. Each row shows data for one infant (n=117). Colours indicate the Sabin sub-type or combination of sub-types detected on that day. Colours do not relate to quantitative shedding level. Undetectable shedding is shaded grey. Missing data, where a stool sample was not provided, is shown in white.

Infants receiving three doses of trivalent OPV, including a birth dose, were included for poliovirus-specific neutralizing antibody analysis. The proportion of these infants with protective poliovirus-specific neutralizing titres prior to 1st rotavirus dose at 6 weeks was 58/85 (68%, 57-71%) for serotype 1 and 126/85 (31%, 22-41%) for serotype 3. The majority of infants attained protective poliovirus-specific neutralizing antibody titres by 12 weeks: 78/85 (92%, 84-96%) for serotype 1 and 71/85 (84%, 74%-90%) for serotype 3. Between 6 and 12 weeks, 37/85 (44%, 33-54%) infants seroconverted to serotype 1 and 51/85 (60%, 49-70%) infants seroconverted to serotype 3.

Protective serotype 3 neutralizing antibody titres at 6 weeks were associated with a 59% reduction in the likelihood of shedding Sabin 3 following the subsequent dose of OPV: RR 0.41 (95%CI 0.22-0.79), p=0.007 (Table 3-7). Protective serotype 1 antibody titres at 6 weeks were associated with a 70% reduction in likelihood of shedding Sabin 1 following the subsequent dose of OPV: RR 0.30 (95%CI 0.16-0.54), p<0.001.

Attainment of protective serotype 1 poliovirus-specific neutralizing antibody titres at 12 weeks was not associated with post-immunisation shedding of the Sabin 1 vaccine virus.

Table 3-7: Sabin 1 virus shedding and type 1 poliovirus neutralizing antibody response

OPV vaccine virus shedding*	Protective type 1 poliovirus neutralizing antibody titres at 12 weeks RR (95%CI)	Type 1 poliovirus neutralizing antibody seroconversion between 6 and 12 weeks RR (95%CI)
Any Sabin 1: 1 st dose period	1.1 (0.96-1.2) p=0.18	3.7 (2.1-6.4) p<0.001
Any Sabin 1: 2 nd dose period	1.0 (0.90-1.2) p=0.61	1.8 (1.2-3.0) p=0.008
Any Sabin 1: either dose period	1.2 (0.98-1.4) p=0.09	7.8 (2.7-23.3) p<0.001

* Shedding data available for 1st dose period n=76, 2nd dose period n=70, either dose period n=64

Attainment of protective serotype 3 poliovirus-specific neutralizing antibody titres at 12 weeks was positively associated with shedding of the Sabin 3 vaccine virus in the 1st dose period at 6 weeks but not with Sabin 3 vaccine virus shedding in the 2nd dose period (Table 3-8).

Table 3-8: Sabin 3 virus shedding and type 3 poliovirus neutralizing antibody response

OPV vaccine virus shedding*	Protective type 3 poliovirus neutralizing antibody titres at 12 weeks RR (95%CI)	Type 3 poliovirus neutralizing antibody seroconversion between 6 and 12 weeks RR (95%CI)
Any Sabin 3: 1 st dose period	1.4 (1.1-1.8) p=0.006	4.3 (2.2-8.4) p<0.001
Any Sabin 3: 2 nd dose period	1.1 (0.94-1.4) p=0.18	1.6 (1.1-2.3) p=0.02
Any Sabin 3: either dose period	1.4 (0.97-2.1) p=0.07	∞ N/A**

*Shedding data available for 1st dose period n=76, 2nd dose period n=70, either dose period n=64

**All 36 infants with serotype 3 seroconversion shed Sabin 3. RR incalculable.

For both serotypes 1 and 3, Sabin vaccine virus shedding following immunisation strongly predicted serotype-specific seroconversion, defined as a change from seronegative to seropositive or a four-fold rise in titres, between weeks 6 and 12 (Table 3-7 and Table 3-8). It should be noted that this definition excludes infants seropositive at baseline who did not have a four-fold rise in concentration. However, those infants seropositive at baseline who did not seroconvert are included in the total number of infants seropositive at 12 weeks.

Sabin vaccine virus shedding during the 1st dose period was more strongly predictive of serotype-specific poliovirus seroconversion between weeks 6 and 12 than shedding during the 2nd dose period.

3.4.6.2 OPV and RV1 vaccine virus shedding

In infants who had received a birth dose of OPV, OPV virus shedding prior to the first rotavirus vaccine dose was associated with a two-fold increase in the likelihood of RV1 shedding in the 1st dose period. A similar, non-significant, trend was seen for infants with OPV shedding prior to the 2nd rotavirus vaccine dose.

OPV shedding overall or by sub-type was not significantly associated with RV1 shedding in the 1st dose period (Table 3-9). In the 2nd dose period, OPV shedding post-dose was associated with a two-fold increase in the likelihood of RV1 shedding. This trend was consistent when looking at

the association between RV1 shedding and shedding by Sabin sub-type. Predominant shedding of a combination of Sabin 1 or 3 was most strongly associated with RV1 shedding in the 2nd dose period.

Table 3-9: Risk ratio for RV1 shedding by OPV shedding

OPV vaccine virus shedding pattern*	RV1 shedding 1 st dose period RR (95%CI)	RV1 shedding 2 nd dose period RR (95%CI)
Any OPV shedding pre-dose	2.0 (1.0-4.3) p=0.04	1.9 (0.98-3.5) p=0.06
Any OPV shedding post-dose	0.80 (0.47-1.4) p=0.40	2.0 (1.0-3.8) p=0.04
Any Sabin 1 combination	0.88(0.49-1.6) p=0.66	2.2(1.1-4.5) p=0.02
Any Sabin 2 combination	0.94 (0.54-1.6) p=0.82	1.8(0.85-4.0) p=0.12
Any Sabin 3 combination	0.85(0.49-1.5) p=0.56	2.3(1.2-4.5) p=0.01

*Paired data available for: Pre-1st dose n=85, post 1st dose n=171, pre 2nd dose n=102, post 2nd dose n=132

The association between OPV shedding pattern and RV1 shedding overall could not be reliably determined by logistic regression, due to the very small number of infants (six) with no OPV shedding overall: 5/6(83%) infants with no OPV shedding shed RV1, compared to 55/111(50%) infants with OPV shedding. This difference was not statistically significant by Fisher's exact test (p=0.21).

There was no evidence of correlation between peak RV1 shedding and peak shedding of either of the three Sabin sub-types following either dose (Table 3-10).

Table 3-10: Correlation between peak shedding of RV1 and Sabin sub-types

	Correlation with RV1 shedding 1 st dose period	Correlation with RV1 shedding 2 nd dose period
Peak shedding Sabin 1	ρ 0.11, p=0.61	ρ -0.51, p=0.05
Peak shedding Sabin 2	ρ -0.04, p=0.85	ρ 0.02, p=0.96
Peak shedding Sabin 3	ρ 0.26, p=0.20	ρ 0.52, p=0.52

ρ = Spearman's rho. p= p value.

There was no difference in peak RV1 shedding between infants with and without OPV shedding after either dose (Wilcoxon rank-sum test, $p=0.85$ and $p=0.83$ for 1st and 2nd dose period respectively).

3.4.6.3 Poliovirus-specific neutralizing antibody response and RV1 vaccine virus shedding

In infants who received three doses of trivalent OPV, including a birth dose, protective titres to poliovirus serotype 3 at 6 weeks of age, prior to 1st rotavirus immunisation, were associated with a two-fold increase in the likelihood of RV1 shedding in the 1st dose period, but were not associated with RV1 shedding in the 2nd dose period (Table 3-11). Protective titres to poliovirus serotype 1 at 6 weeks were not associated with RV1 shedding. For both poliovirus types 1 and 3, neither protective neutralizing antibody titres at 12 weeks, nor seroconversion between 6 and 12 weeks, were associated with RV1 shedding.

Table 3-11: Poliovirus-specific neutralizing antibody and RV1 shedding

Poliovirus-specific neutralizing antibody response*	RV1 shedding 1 st dose period RR 95%CI	RV1 shedding 2 nd dose period RR 95%CI	RV1 shedding either dose period RR 95%CI
Protective titre at 6 weeks			
Poliovirus 1	1.2 (0.60-2.5) $p=0.59$	0.81 (0.36-1.8) $p=0.60$	0.95 (0.58-1.6) $p=0.88$
Poliovirus 3	2.24 (1.25-4.0) $p=0.007$	1.54 (0.71-0.34) $p=0.28$	1.7 (1.1-2.6) $p=0.02$
Protective titre at 12 weeks			
Poliovirus 1	1.3 (0.37-4.2) $p=0.72$	0.71 (0.21-2.4) $p=0.58$	0.97 (0.42-2.2) $p=0.94$
Poliovirus 3	2.8 (0.74-10.4) $p=0.13$	1.9 (0.48-7.1) $p=0.37$	1.9 (0.79-4.4) $p=0.16$
Seroconversion			
Poliovirus 1	0.92 (0.49-1.7) $p=0.79$	0.89 (0.40-2.0) $p=0.78$	1.1 (0.65-1.7) $p=0.21$
Poliovirus 3	0.81 (0.44-1.5) $p=0.49$	0.82 (0.37-1.8) $p=0.61$	0.95 (0.59-1.5) $p=0.83$

*Paired data available for: 1st dose period $n=77$, 2nd dose period $n=78$, Both dose periods $n=72$

3.4.7 HBGA phenotype and RV1 vaccine virus shedding

The distribution of HBGA phenotype in the shedding cohort is shown in Table 3-12.

*Table 3-12: Distribution of secretor and Lewis negative phenotype in shedding cohort**

	Lewis positive	Lewis negative	Total
Non-secretor	30	15	45 (22%)
Secretor	129	28	157(78%)
Total	159 (79%)	43 (21%)	202

**Distribution is given for infants with shedding data available for both dosing periods. Distribution of HBGA phenotype was similar in infants with shedding data for only the 1st or 2nd dose period.*

FUT2 genotyping was unavailable for 15/45 infants who were phenotypic non-secretors at 6 weeks old as they had insufficient whole blood samples for testing. The remaining thirty phenotypic non-secretors completed FUT2 genotyping. Of these infants, 27 were confirmed as non-secretor genotype. Three infants were confirmed as heterozygous secretors, two of these infants had a secretor salivary phenotype at 10 weeks old and one remained a phenotypic non-secretor. Of the 15 infants who were phenotypic non-secretors at 6 weeks old who did not have FUT2 genotyping available, four were phenotypic secretors at 10 weeks old.

The proportion of infants with saliva positive for both Le^a and Le^b was high: 90/202 (45%, 38-50%) were Le^{a+b}. Most of these infants were phenotypic secretors: 79/157 (50%, 42-58%) secretors and 11/45(24%, 14-40%) non-secretors were Le^{a+b+}. All eleven Le^{a+b+} non-secretor infants were confirmed as non-secretors by FUT2 genotyping. A further 15/45(33%, 23-48%) non-secretor infants were Le^{a+b-} and 15/45(33%, 23-48%) were Lewis negative (Le^{a-b-}).

Unexpectedly, 4/45(9%) phenotypic non-secretors were Le^{a-b+}. Three of these infants were confirmed as non-secretors by FUT2 genotyping. One had no blood available for genotyping, but had secretor salivary phenotype at 10 weeks old.

In an initial unstratified analysis, there was weak evidence that secretor phenotype was associated with increased likelihood of RV1 vaccine virus shedding following the 1st dose RV1, but no association after the second dose or overall (Table 3-13). There was no significant association between secretor phenotype and vaccine virus shedding overall: 18/45 (40%, 26-

55%) non-secretor infants had RV1 shedding overall, compared to 86/157 (55%, 47-62%) secretor infants (χ^2 p=0.08).

There was no association seen between Lewis negative phenotype and likelihood of shedding after either dose and no association overall: 20/43 (47%, 32-62%) Lewis negative infants had RV1 shedding overall, compared to 84/159 (53%, 45-61%) Lewis positive infants (χ^2 p=0.46).

Table 3-13: Relationship between HBGA phenotype and RV1 shedding

	Secretor RR (95%CI)	Lewis negative RR (95%CI)
RV1 shedding after 1 st dose n=73/243	1.8 (1.0-3.3) p=0.04 n=63/188	0.92 (0.56-1.5) p=0.73 n=14/50
RV1 shedding after 2 nd dose n=70/214	1.8 (0.87-3.7) p=0.11 n=62/178	0.86 (0.52-1.4) p=0.55 n=8/36
RV1 shedding after either dose n=104/202	1.4 (0.93-2.0) p=0.11 n=86/157	0.88 (0.62-1.3) p=0.48 n=20/43

Since secretor and Lewis phenotype are determined by the combination of FUT2 and FUT3 gene expression there is significant overlap between these phenotypes. To determine whether the combination of Lewis and secretor phenotype modified the likelihood of vaccine virus shedding, secretor and non-secretor infants were stratified by Lewis phenotype (Table 3-14). Stratification did not significantly modify the effects observed in unstratified analysis.

Table 3-14: RV1 vaccine virus shedding by stratified secretor and Lewis phenotype

HBGA phenotype	RV1 shedding after either dose n, %, RR (95%CI)
<u>Non-secretor</u>	n=18/45 (40%) 0.73(0.50-1.1) p=0.11
<i>Lewis negative</i>	n=8/15 (53%) 1.6 (0.8-3.2) p=0.18
<i>Lewis positive</i>	n=10/30 (33%) 0.6 (0.3-1.2) p=0.18
<u>Secretor</u>	n=86/157 (55%) 1.4 (0.93-2.0) p=0.11
<i>Lewis negative</i>	n=12/28 (43%) 0.8 (0.5-1.2) p=0.21
<i>Lewis positive</i>	n=74/129 (57%) 1.3 (0.9-2.1) p=0.21

To determine the relationship between HBGA phenotype and quantitative shedding, peak shedding (defined as the reciprocal of the minimum NSP2 Ct value) was compared by Lewis/secretor phenotype. There was no significant difference in median Ct value for peak shedding between secretors and non-secretors, or between Lewis positive and negative infants for either dose period.

Table 3-15 Median Ct Value for peak shedding by HBGA phenotype

Median Ct value (IQR) for peak shedding (1st dose period)	Median Ct value (IQR) for peak shedding (2nd dose period)
Secretor 29.3 (26.0-32.3) vs Non-secretor 31.9 (30.4-34.1) p=0.13	Secretor 32.4 (30.6-34.6) vs Non-secretor 34.1 (31.9-35.0) p=0.21
Lewis positive 29.8 (26.4-32.4) vs Lewis negative 31.2(28.0-34.1) p=0.41	Lewis positive 32.1 (30.6-34.7) vs Lewis negative 33.9 (32.7-35.4) p=0.15

vs= comparison by Mann Whitney test

Additional analyses

RV1 shedding was compared by Le^{a+b-} phenotype. There was no difference in RV1 shedding after either dose in Le^{a+b-} infants compared to infants with other Lewis phenotype: 12/25 (48%, 28-68%) with RV1 shedding compared to 92/177 (52%, 45-59%) respectively (χ^2 p=0.71).

There was no difference in the proportion of infants with RV1 shedding after either dose by ABO phenotype (Table 3-16).

Table 3-16: RV1 vaccine virus shedding by ABO type

ABO Type	O	A	B	AB	p value*
RV1 shedding after either dose	35/67, 52% (40-64%)	28/45, 62% (47-76%)	20/37, 54% (37-70%)	3/8 (38%) (9-79%)	0.54

*Fisher's exact test

3.4.8 Demographic and socioeconomic factors

The relationships between demographic and socioeconomic factors and vaccine virus shedding are summarized in Table 3-17. There was no association between male sex, low birth weight or HIV exposure and RV1 vaccine virus shedding.

There was some evidence that both weight for age and MUAC at time of 1st dose were positively associated with vaccine virus shedding. A 1cm increase in MUAC or 1 standard deviation increase in weight for age was associated with around a 20% increase in overall likelihood of RV1 vaccine virus shedding.

No proxy measure of overcrowding or sanitation was associated with RV1 vaccine virus shedding. The only proxy measure of poverty associated with shedding was years of maternal education. For every one-year increase in maternal education, there was a 5% increase in likelihood of RV1 vaccine virus shedding.

Table 3-17: Demographic and socio-economic predictors of RV1 vaccine virus shedding

Characteristic	Shedding after 1 st dose RR (95%CI)	Shedding after 2 nd dose RR (95%CI)	Shedding after either dose RR (95%CI)
<u>Infant characteristics</u>			
Male	0.85 (0.58-1.3) p=0.42	1.1(0.72-1.5) p=0.77	1.0 (0.77-1.3) p=1.0
HIV-exposed	0.70 (0.35-1.4) p=0.31	0.50(0.22-1.1) p=0.09	0.59(0.34-1.0) p=0.06
Low birth weight (<2.5kg)	0.71 (0.32-1.6) p=0.40	0.75(0.36-1.5) p=0.43	0.71(0.41-1.3) p=0.24
<u>Nutritional status</u>			
Weight for age z-score	1.1(0.90-1.3) p=0.38	1.0(0.83-1.2) p=0.97	1.2 (1.0-1.3) p=0.02
Length for age z-score	1.0 (0.87-1.2) p=0.88	0.95(0.81-1.1) p=0.47	1.1(0.99-1.2) p=0.07
Median weight for length z-score	1.0 (0.90-1.2) p=0.68	1.1(0.96-1.29) p=0.17	1.0(0.94-1.1) p=0.62
MUAC, cm	1.1 (0.91-1.4) p=0.31	1.1(0.88-1.3) p=0.56	1.2 (1.0-1.3) p=0.02
<u>Sanitation and socioeconomic predictors</u>			
Household size	0.97 (0.85-1.1) p=0.65	1.0(0.88-1.1) p=0.97	1.1(0.97-1.1) p=0.23
Non-piped water source	0.57 (0.25-1.3) p=0.18	1.4(0.83-2.3) p=0.21	0.87(0.54-1.4) p=0.57
Time taken to access water			
<5minutes	ref	ref	ref
5-30 minutes	1.4(0.91-2.2) p=0.13	1.3 (0.85-1.9) p=0.24	1.1 (0.85-1.5) p=0.41
>30 minutes	1.3(0.66-2.8) p=0.41	0.32(0.08-1.2) p=0.10	0.71 (0.37-1.3) p=0.29
Pit-latrline type toilet	0.94 (0.49-1.8) p=0.84	0.94(0.50-1.8) p=0.85	0.73(0.52-1.0) p=0.07
Electricity at home	1.3 (0.88-1.9) p=0.18	0.80(0.54-1.2) p=0.25	1.2 (0.89-1.5) p=0.27
One or more household members with salary	1.0 (0.63-1.7) p=0.90	0.95(0.59-1.5) p=0.83	0.95(0.68-1.3) p=0.74
Household food insecurity	0.81 (0.52-1.3) p=0.35	0.98(0.65-1.5) p=0.93	0.92 (0.68-1.2) p=0.57
Age of head of household, years	1.0(0.99-1.0) p=0.16	0.99(0.98-1.0) p=0.53	1.0 (0.99-1.0) p=0.36
Years of maternal education	1.1(0.98-1.1) p=0.16	1.0(0.95-1.1) p=0.90	1.05 (1.0-1.1) p=0.03

3.4.9 Multivariable analysis of predictors of RV1 vaccine virus shedding

All variables predicting overall RV1 shedding (RV1 shedding after either dose) significant to $p < 0.10$ in bivariable analysis were considered for inclusion in multivariable analysis (Table 3-18). Where variables were co-linear, only the variable with the lowest p value was tested initially, and other significant factors tested separately. Manual stepwise backwards elimination was used. At each step, the variable with the highest p value was eliminated until only variables with p values < 0.10 remained.

Poisson regression with robust errors was used as a binomial model failed to converge. As expected, the anthropometric measures weight for age, MUAC and length for age were highly collinear and therefore tested separately. Only log RV-specific IgG and weight for age z score remained significant predictors of RV1 vaccine virus shedding in multivariable analysis. The Akaike Information Criterion (AIC) statistic was used to compare each iteration of the model and confirmed that the final model provided the best fit.

Table 3-18: Predictors of RV1 vaccine virus shedding: multivariable analysis

Predictor variable	Univariable analysis IRR (95%CI)	Multivariable analysis IRR (95%CI)
Log RV-specific IgG	0.81(0.72-0.92) $p=0.001$	0.82 (0.73-0.93) $p=0.002$
Weight for age z score	1.2 (1.0-1.3) $p=0.02$	1.1 (1.0-1.3) $p=0.04$
MUAC, cm	1.2 (1.0-1.3) $p=0.03$	NA
Years of maternal education	1.05 (1.0-1.1) $p=0.04$	NA
HIV exposed	0.59 (0.34-1.0) $p=0.07$	NA
Length for age z score	1.1 (0.99-1.2) $p=0.07$	NA
Pit latrine type toilet	0.73 (0.52-1.0) $p=0.07$	NA

NA= not applicable -not significant in multivariable analysis

3.5 DISCUSSION

RV1 vaccine virus shedding in Malawian infants following RV1 immunisation at 6 and 10 weeks

My study provides the first data on RV1 vaccine virus shedding in Malawian infants. Overall RV1 vaccine virus shedding rates in this population were 30% (25-36%) following 1st dose RV1, 33% (27-39%) after the 2nd dose RV1 and 52% (45-58%) after either vaccine dose. Shedding rate following the 1st dose was similar to that reported in urban Vellore, where 24% of infants shed rotavirus on day 4 or 7 following 1st RV1 vaccination at 6 weeks (Lazarus et al., 2017).

Most other published studies of vaccine virus shedding have used rotavirus antigen detection using enzyme immunoassay (EIA). This method is less sensitive than RT-PCR and is less likely to detect low-level shedding. A small study comparing shedding of monovalent and pentavalent vaccine in Taiwanese infants using both RT-PCR and EIA found detection rates of 80-90% using RT-PCR, and only 20-30% using EIA (Hsieh et al., 2014). Level of shedding was low in my cohort study particularly following the 2nd dose, with median Ct values of 29.9 and 32.7 following the 1st and 2nd doses respectively. Much of this shedding would have been missed by EIA – in the cohort using a Ct cut-off of 27 to approximate EIA positivity overall shedding rates dropped from 52% to 11%. This may explain why shedding rates determined by EIA reported in lower middle income countries such as Bangladesh (25% overall) and Ghana (5% overall) were so low (Zaman et al., 2009, Armah et al., 2016) compared to rates reported in higher income countries (rates of 35-80% following the 1st dose in pre-licensure studies (Anderson, 2008)).

Although direct comparison with shedding rates in published studies in other populations is problematic due to these methodological differences, comparison of overall shedding patterns is instructive. In most studies from higher income countries, the majority of shedding occurs following the 1st dose with little shedding following dose 2. Vesikari et al. (2004) in Finland reported shedding rates of 52% following the 1st dose, and only 7% following the 2nd dose. Similarly, Phua et al. (2005) in Singapore reported shedding of 76-80% after 1st dose, compared to 18-29% post dose 2.

In studies from middle income countries in Latin America, the decline in shedding following dose 2 was less steep: Salinas et al. (2005) in a study recruiting from Brazil, Mexico and Venezuela reported shedding rates of 35-44% following the 1st dose and 11-21% following the 2nd dose. In a further study in Mexico, Ruiz-Palacios et al. (2007) reported very similar rates.

The shedding pattern in this low-income Malawian population was quite different to the pattern described in high income countries above, with broadly equal numbers of infants shedding following the 1st or 2nd doses, and 10% of infants shedding following both doses.

How can we explain these differences between and within populations and how might this improve our understanding of how shedding patterns might relate to vaccine response?

Figure 3-6 presents a conceptual framework by which vaccine virus shedding might relate to vaccine response. In this framework, we consider infants in four groups, characterized as early responders, late responders, low shedders and persistent shedders.

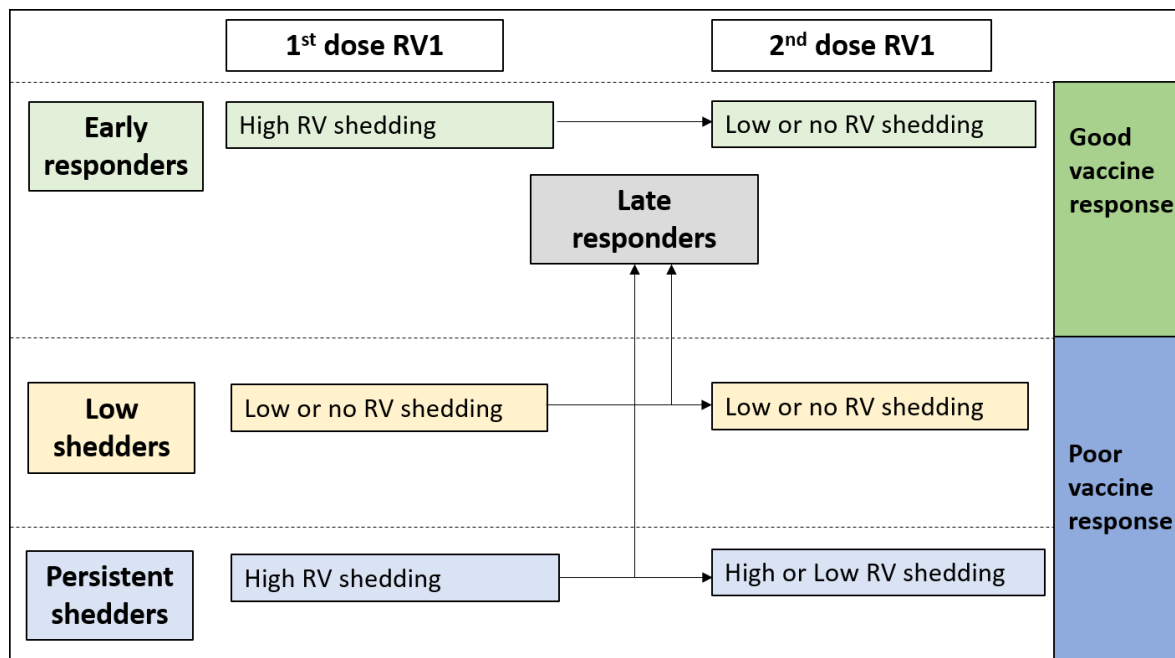


Figure 3-6: Conceptual framework of RV vaccine virus shedding

Early responders are the optimal responders – infants who show robust vaccine virus replication which generates an effective initial and subsequent mucosal immune response. In this group, we would predict high vaccine virus shedding for initial doses, but low or no vaccine

shedding for subsequent doses. Very effective early response might be demonstrated by short duration shedding, even to the first dose, reflecting rapid immune control of vaccine virus replication. This fits with the pattern of shedding rates reported in high income countries – high following the 1st dose and low following the second, with high vaccine efficacy (Anderson, 2008).

Late responders are infants who have delayed shedding or an ineffective immune response to the 1st dose, but more effective response following the 2nd dose. They might start in either of the poor response groups at dose 1, but the removal of an inhibitory factor would allow them to develop an appropriate response to subsequent doses. Examples of transient inhibitory effects would be declining maternal antibody, or enteric co-infection. Overall protection in this group would be dependent on the effectiveness of the response to the second dose, but is likely to be lower than the early responders. In the cohort, peak shedding was lower in infants who shed only following the 2nd dose, compared to those who shed only following the 1st dose. Middle income countries, such as those in Latin America, could also have a significant proportion of the population falling within this group, particularly in parts of the population with a higher burden of rotavirus and enteric infection. This might explain the higher proportion of infants shedding following the 2nd dose in these countries, compared to higher income settings (Salinas et al., 2005, Ruiz-Palacios et al., 2007).

Low shedders are infants who show no or low-level vaccine virus replication, and consequently do not stimulate an adequate mucosal immune response. Low vaccine virus replication might arise from an unfavourable gut environment, for example infants with concurrent enteric infection, from genetic factors such as HBGA phenotype, or from inhibition from high levels of maternal antibody. A large proportion of infants in the cohort, and those in populations in lower middle income Bangladesh and Ghana who reported low shedding rates (Zaman et al., 2009, Armah et al., 2016), might fall into this group.

Finally, persistent shedders are infants who show unrestricted vaccine virus replication secondary to an ineffective mucosal immune response. In the cohort, 9% of infants were still shedding RV1 28 days following the 1st vaccine dose, and 10% of infants shed RV1 following

both doses. Persistent shedding might indicate a dysfunctional mucosal immune response (Levine and Robins-Browne, 2012). An example of this would be environmental enteropathy, where chronic immune activation and mucosal immune dysfunction might interrupt the normal pathways of the innate and adaptive anti-viral immune response (Serazin et al., 2010).

My study is unique in using high sensitivity diagnostics and multiple time points to investigate in detail the RV1 shedding response in a low-income setting. Data collection on alternate days for 10 days post vaccine allowed me to identify the shedding time-point most predictive of overall shedding rates as day 6. Supporting this finding, Yen et al. (2011) in a US study of shedding over nine days following the pentavalent rotavirus vaccine also identified day 6 as the time point with peak numbers of shedding, and Hsieh et al. (2014) found viral shedding loads were highest at day 6-7. Given the logistic challenges of collecting stool at multiple time points, these data should better inform future vaccine studies in similar populations to select optimal timing of stool sample collection.

Wild type rotavirus at time of immunisation

Thirty percent of infants with pre-immunisation stool samples at 6 weeks and 13% at 10 weeks were positive for wild-type rotavirus infection. Since vaccine virus shedding and wild-type shedding could be differentiated by the vaccine-specific NSP2 RT-PCR, these infants were not excluded from the study. Most were asymptomatic and reported no recent history of diarrhoea. Only one child was reported to have diarrhoea severe enough to require hospital admission in the first six weeks of life. This supports findings of previous studies in other settings suggesting that neonatal rotavirus is common, but generally asymptomatic. This is of public health importance when considering the potential benefits of neonatal rotavirus vaccines in this setting. There are few published data on community acquired rotavirus infection in early infancy in Malawi. A previous study of rotavirus infection in a secondary care neonatal unit in Blantyre found a prevalence of 25%, but risk and exposure are likely to vary in a hospital setting (Cunliffe et al., 2002). Early infant exposure to rotavirus was estimated at 10% based on rotavirus specific IgA seropositivity at 6 weeks in the Malawi vaccine trial, but this may underestimate infection (Cunliffe et al., 2014). In the cohort, stool positivity (30%) was higher

than seropositivity (10% - see chapter 4) at 6 weeks of age. One possible explanation would be that wild-type neonatal rotavirus infections may not generate a robust mucosal immune response. Epidemiological evidence supports this hypothesis, since early infant infection does not protect against future disease in high burden settings (Gladstone et al., 2011). This could potentially be due to several reasons including inhibition by maternal antibody, developmental immaturity of infant mucosal immunity, lower immunogenicity of neonatal rotavirus genotypes or differences in the infant microbiome. Further research in this area is needed to better understand the nature of, and response to, neonatal rotavirus infections. This could have important implications for the effectiveness of neonatal rotavirus vaccines.

Wild-type infection at time of immunisation could have contributed to RV-specific IgA seroconversion during the immunisation period in some infants, this is discussed further in Chapter 4. Possible limitations of the IgA ELISA assay used are also discussed in Chapter 4.

Relationship between RV-specific IgG and RV1 vaccine virus shedding

We confirmed the hypothesis that higher levels of passively-acquired maternal rotavirus antibody (RV-specific IgG) at time of 1st RV1 immunisation would be associated with lower RV1 vaccine virus shedding, however this inhibitory effect was only significant for shedding following the 1st dose. Following the 2nd dose, there was only evidence of an inhibitory effect at the highest baseline levels of RV-specific IgG. There was some evidence of a weak negative correlation between RV-specific IgG and RV1 vaccine virus shedding, but stronger evidence for an inhibitory association at the highest IgG quartile. Taken together, these observations suggest a threshold effect, where only RV-specific IgG above a certain level is associated with significant inhibition of RV1 vaccine virus shedding. This would explain why the inhibitory effect has diminished by the 2nd dose, since the decline of maternal antibody would predict that only infants with the highest baseline levels of passively-acquired IgG would remain above the inhibitory threshold at 10 weeks.

While other studies have reported an inhibitory effect of passively-acquired RV-specific IgG on RV1 IgA response, we believe this is the first study to report a negative association between RV-specific IgG and rotavirus vaccine virus shedding. Evidence from a primate study of the effect of

passive immunization on rotavirus shedding suggests a potential mechanism. Westerman et al. (2005) passively immunised naïve infant pigtailed macaques with high or intermediate titre RV-specific IgG, and measured virus shedding following a simian rotavirus challenge. Passively immunized primates showed no or delayed, low level shedding compared to controls. RV-specific IgG could also be detected in stools of primates immunized with high titre serum. The authors suggested a possible mechanism of this inhibitory effect could be that during transcytosis of excess IgG into the intestinal lumen, IgG could prevent rotavirus attachment or block replicative steps of rotavirus within the intestinal endothelial cell. A mechanism such as this, requiring high titre IgG to mediate an effect, could explain the threshold effects observed in the cohort. Further mechanistic studies are required to confirm this proposed mechanism.

An alternative explanation is that the negative association between serum RV-specific IgG and vaccine virus replication may be indirect, rather than causal. Almost all infants in the cohort were breast-fed. Breast milk contains high concentrations of IgA which can be measured in expressed samples (Weaver et al., 1998). Previous studies have demonstrated in-vitro rotavirus neutralizing effects of maternal IgA and other factors in breast-milk (Moon et al., 2010, Moon et al., 2013, Glass and Stoll, 1989). Since maternal IgA and IgG are both associated with rotavirus exposure, it seems likely they may be correlated. High maternal IgG may therefore be a proxy marker for high maternal breast-milk IgA, which may be the primary inhibitor of vaccine virus replication in the infant gut.

However, there is also evidence that the proposed inhibitory effect of breast-feeding on rotavirus vaccine virus response may have been overemphasized. Results from epidemiological studies determining the effect of breast-feeding on response to older rotavirus vaccine were equivocal (Glass et al., 1991), and suggested any inhibition was overcome by the 2nd vaccine dose. A recent observational study in Mexico reported reduced vaccine responses in breast-fed infants, but did not report or adjust for other socio-economic differences between breast-fed and non-breast-fed infants (Bautista-Marquez et al., 2016). Three large randomized controlled trials of withholding breastfeeding at time of immunization have shown no benefit (Groome et al., 2014, Ali et al., 2015, Rongsen-Chandola et al., 2014). In addition, Moon et al. (2016) in South Africa showed no effect of maternal breast milk IgA levels on RV1 immunogenicity, but

did observe an inhibitory effect of maternal IgG. In this context, direct effects of RV-specific IgG on vaccine virus shedding remain plausible. Further studies which could determine correlations between maternal IgA, maternal IgG and their individual or combined effects on rotavirus vaccine virus shedding would help resolve this issue. The ongoing RoVi study in Malawi, India and the UK which will determine both maternal breast milk and serum antibodies and infant rotavirus vaccine response, should prove informative (Sindhu et al., 2017).

What would be the implications of a direct effect of maternal RV-specific IgG on rotavirus replication? Siegrist (2003) suggests that the inhibitory effect of maternal antibody on vaccine virus replication will depend on the ratio between maternal antibody and vaccine virus load/replicating capacity. This might also be true of the protective immunity offered by maternal IgG, predicting protection, particularly against low infective doses, in early infancy when IgG is highest, but rapidly declining protection once IgG falls below the threshold ratio. This fits the observed natural history of rotavirus infection in infancy. Protection could be reduced in circumstances where maternal IgG might be relatively low, or infective dose high – for example in an outbreak of rotavirus in a neonatal unit in London where maternal IgG did not appear to be protective against rotavirus infection (Totterdell et al., 1980). Dependence on a maternal antibody/viral load ratio would also predict that inhibitory effects of maternal antibody could be overcome by increasing doses of live vaccine virus, as was observed in dosage studies of the Indian rotavirus vaccine ORV-116E (Appaiahgari et al., 2014).

A threshold, rather than linear, inhibitory effect of maternal antibody has important implications for vaccine scheduling. A linear inhibitory effect would predict that vaccine response in populations with higher IgG at baseline would always be sub-optimal in comparison to populations with lower IgG at any age up to 12 months when maternal antibody disappears. Delaying vaccination would result in an improved, but still sub-optimal, response. A threshold effect predicts that delaying vaccination, or adding booster doses, at a time point where maternal IgG has fallen below the threshold of inhibition, would result in an optimal response equivalent to low IgG baseline populations. However, it also predicts that in populations with very high baseline IgG, booster doses will have no effect until maternal antibody declines below the threshold. This is discussed further in Chapter 4.

Relationship between OPV vaccine response and RV1 vaccine virus shedding

We found no evidence to support the hypothesis that OPV virus shedding at time of rotavirus immunization is associated with lower RV1 vaccine virus shedding. On the contrary, OPV shedding in the 2nd dose period was associated with increased likelihood of RV1 shedding.

Although other studies have examined the effect of OPV in stool prior to 1st rotavirus immunization on RV1 shedding, I believe this is the first study to compare OPV and RV shedding concurrently throughout the dosing period. I found no evidence of a quantitative relationship between OPV and RV1 shedding: there was no correlation between peak RV1 vaccine shedding and peak shedding of any Sabin sub-type, and peak RV1 vaccine virus shedding did not differ between infants with and without concurrent OPV shedding. Although these findings may be limited by small numbers and overall low-level shedding, these data would seem to contradict the hypothesis that inhibitory effects of concurrent OPV immunization on rotavirus vaccine response are mediated through direct competition for vaccine virus replication in the gut. This does not exclude an inhibitory effect of concurrent OPV via alternative immune-mediated mechanisms (see Chapter 4).

The inter-relationship of OPV and RV1 vaccine virus shedding may best be understood by referring again to the conceptual framework for vaccine virus shedding introduced in Figure 3-6, but now adding response to OPV from birth dose onwards (Figure 3-7).

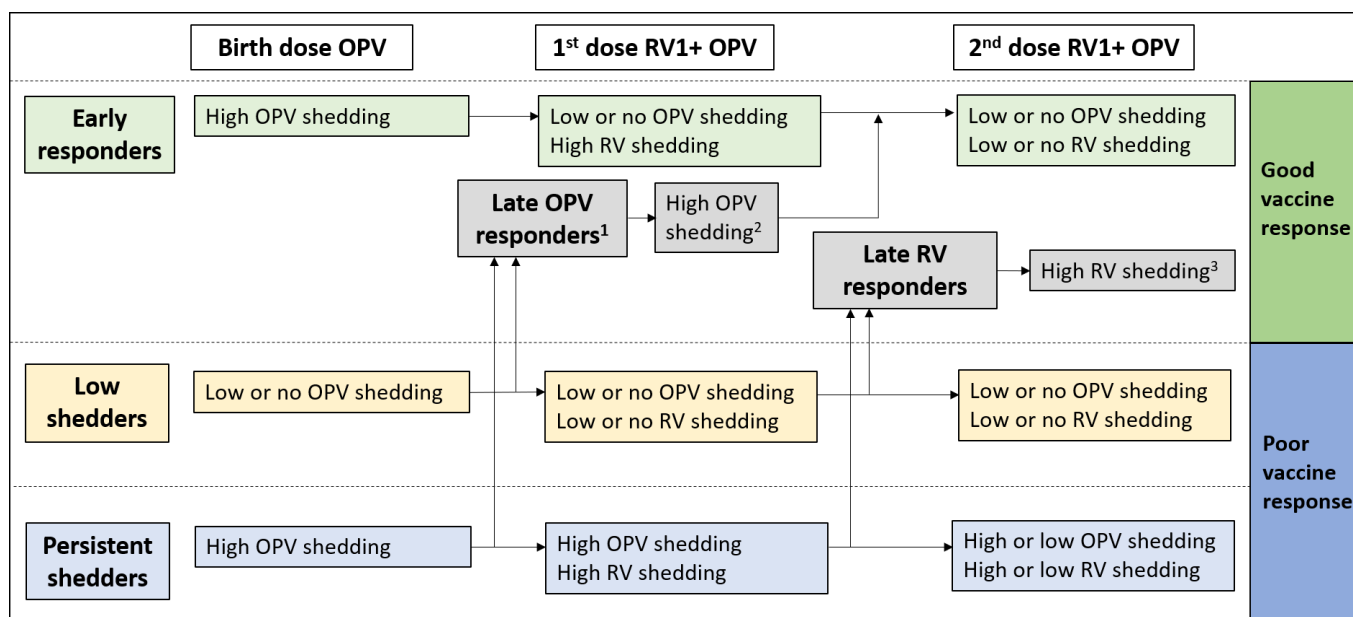


Figure 3-7: Conceptual framework of OPV and RV1 vaccine response

1. Late OPV response may also be delayed until dose 3 or later 2. RV shedding in late OPV responders may be variable, dependent on any inhibitory factor and its effect on RV1 response 3. OPV shedding in late RV responders may be similarly variable.

The pattern of OPV shedding we observed by Sabin sub-type supports the concept of “early responders” where robust immune response to earlier doses will reduce subsequent vaccine virus shedding to that serotype. For example, Sabin 2 was the predominant serotype shed at baseline, six weeks following the birth dose of tOPV, but progressively less Sabin 2 shedding was observed following subsequent doses. Similarly, predominant Sabin combinations in the 1st dose period were rarely repeated in the 2nd. Further confirming this concept is my observation that in infants receiving a birth dose of tOPV, protective poliovirus-specific neutralizing antibody titres at 6 weeks were associated with a 60-70% decrease of in the likelihood of shedding the corresponding Sabin vaccine virus following subsequent immunisation.

My data also provides supportive evidence for the concept of “late responders”, where removal of a transient inhibitory factor allows delayed but effective vaccine response. For example, Sabin 2 vaccine virus is known to inhibit the replication of Sabin 3. This was confirmed in the cohort study, where concurrent shedding of Sabin 2 and 3 subtypes was uncommon. The lower proportion of infants with protective poliovirus-specific neutralizing antibody titres to type 3 poliovirus at observed 6 weeks are likely to reflect inhibition by Sabin 2 at the birth dose. The

decline in Sabin 2 shedding with subsequent doses, as described above, allows a more robust Sabin 3 response to later doses, reflected in the observed high rates of seroconversion to type 3 poliovirus between weeks 6 and 12.

The presence in the population of both early and late responders may explain why type-specific OPV shedding was strongly associated with poliovirus-specific seroconversion, but not with attainment of protective poliovirus-specific neutralizing antibody titres at 12 weeks. The proportion of infants with protective titres at 12 weeks includes both infants who responded early, with protective titres at 6 weeks, and those who responded to later doses. Early responders will have low OPV shedding, and late responders high, bringing the association with protective titres toward the null. The definition of seroconversion largely excludes those with protective titres at 6 weeks, and therefore includes more late responders, with associated higher rates of serotype-specific poliovirus shedding.

Common factors determining oral vaccine response may explain the relationships observed between OPV response and RV1 vaccine virus shedding. For example, contrary to my initial hypothesis, a robust immune response to OPV, reflected by protective neutralizing antibody titres to poliovirus 3 at 6 weeks, was associated with increased likelihood of RV1 shedding in the 1st dose period. Since Sabin 3 is known to be the least immunogenic subtype of trivalent OPV, infants who develop a protective response to a single vaccine dose may be the archetypal early responders, and may also be expected to respond well to other oral vaccines such as rotavirus. An alternative explanation for this observed association would be that since protective titres to type 3 poliovirus are associated with reduced Sabin 3 shedding, this may result in reduced competitive inhibition of RV1 vaccine virus replication. However, this explanation is contradicted by my data which show no evidence that OPV vaccine virus shedding directly inhibits RV1 shedding.

Similarly, common inhibitory factors to enteric virus replication such as host factors, co-infection or unfavourable microbiome might predict infants would be “low-shedders” with sub-optimal vaccine response to both OPV and RV1. For example, Taniuchi et al. (2016) reported that non-polio enterovirus infection at time of immunization was associated with reduced

vaccine response to both OPV and RV. There is certainly epidemiological evidence to support an association in poor response to enteric vaccines, with population efficacy of OPV and rotavirus vaccines following a similar gradient of high to low response in high to low income countries (Serazin et al., 2010).

So why is there not perfect correlation between OPV and RV1 vaccine virus shedding? This is where we need to consider two groups: late responders and persistent shedders.

In late RV responders, transient inhibitory factors might be absent at birth, allowing an effective initial OPV vaccine response, but present at subsequent doses, therefore having a greater inhibitory effect on RV vaccine response. An example might be non-polio enterovirus infection, as discussed above. In addition, some inhibitory factors might have greater effect on RV than OPV vaccine response. For example, evidence suggests that maternal antibody has little inhibitory effect on OPV response (Bavdekar et al., 1999). Transient or variable inhibitory effects could contribute to a discordant OPV and RV vaccine response in this group, with associations in either direction.

In infants characterized as persistent shedders, persistent OPV shedding following multiple doses reflects failure of an effective mucosal immune response. Common factors such as immune dysfunction related to environmental enteropathy or micronutrient deficiency might determine that infants will respond similarly ineffectively to both OPV and RV1. The concept of persistent shedders would predict that infants in this group would shed both OPV (potentially several Sabin sub-types) and RV1 in the 2nd dose period and beyond.

There is some evidence to support the concept of persistent shedders within the study data. Shedding of Sabin 1 and 3 in the 2nd dose period was much less strongly associated with poliovirus-specific immune response at 12 weeks than shedding in the 1st dose period. Infants who shed following both doses were more likely to shed multiple Sabin sub-types, with a persistence of Sabin 2.

The concept of persistent shedders could explain why RV1 shedding was positively associated with both OPV shedding at 6 weeks, and OPV shedding in the 2nd dose period. Persistent shedding following the birth dose, or shedding of OPV in the 2nd dose period (following the 3rd

dose of OPV for most infants) may represent a sub-optimal OPV vaccine response. Infants with persistent OPV shedding may show similar shedding patterns following rotavirus immunisation, leading to a positive, but not causal, association. A positive association between pre-dose OPV shedding and subsequent RV1 shedding was also observed by Parker et al. (2018) in infants in Vellore. The authors suggested that the positive effect on RV shedding was due to reduced inhibition by OPV, due to lower OPV vaccine take. However, Parker et al (2018) did not directly compare OPV and RV1 shedding during the post-immunization period, and since the current study data suggest no direct competitive inhibition for replication or quantitative relationship between RV1 and OPV, this hypothesis appears less likely to be the case.

This conceptual framework would predict that overall relationships between OPV and RV1 shedding will vary between each dose dependent on the proportion of each of the four groups within a population. For example, during the 1st dose period a balance of early responders (negative association, low OPV associated with high RV shedding), late responders (association in either direction dependent on transient factors), low shedders (positive association, low OPV associated with low RV shedding), and persistent shedders (positive association, high OPV associated with high RV shedding) could bring the overall association toward the null. This might explain the overall null relationship between OPV and RV1 shedding following the 1st dose observed in the cohort.

By the 2nd dose, the negative association between OPV and RV1 shedding in the early responders should disappear, since an effective response to 1st RV1 dose would predict low or no shedding for both RV1 and OPV at that stage. Positive associations in the low shedders and persistent shedders would therefore predominate. This would explain the overall positive association between OPV and RV1 shedding following the 2nd dose observed in the cohort.

Several previous studies have shown an association between concurrent OPV and reduced RV1 immunogenicity (summarized in detail in Chapter 1) but very few studies have reported vaccine virus shedding. One pre-licensure study in Bangladesh examined the effect of concurrent versus staggered administration of OPV and RIX4414 (Zaman et al., 2009) on RV1 vaccine virus shedding (detected by EIA) in a small subset. RV shedding rates were lower in infants receiving

concurrent OPV (6/34, 17.6% (95%CI 6.8-34.5%)) compared to those who received the vaccines 15 days apart (13/42, 31%, 17.6-47.1%) but this difference was not significant.

Due to the switch from trivalent to bivalent OPV during the study period, the sample size for OPV analysis was unavoidably limited. Larger studies are required to confirm some of my key concepts. Restrictions on laboratory use of live poliovirus 2 following the global switch also precluded testing serology for this subtype. Due to the complex interactions of Sabin vaccine responses, my data on trivalent OPV response may differ to responses to bivalent OPV, the new global vaccine. However, the same principles are likely to apply.

A limitation of my study design is that almost all infants received concurrent OPV according to the national schedule. This did not permit comparison of RV vaccine response with and without OPV exposure. The focus of my study was instead on the inter-relationship of OPV and RV vaccine response in a population where both are given concurrently, as remains the case in most low-income settings. These complex relationships, and implications for vaccine strategies, will be expanded on in relation to immunogenicity in Chapter 4. More detailed cohort studies in different populations are required to further explore the balance of these effects.

Relationship between HBGA phenotype and RV1 vaccine virus shedding

My initial hypothesis was that non-secretor and Lewis negative phenotypes would be associated with lower vaccine virus shedding compared to secretors/Lewis positive phenotypes following immunization with the G1P[8] based monovalent rotavirus vaccine. My data provide little evidence to support this hypothesis. There was weak evidence that non-secretor status was associated with reduced likelihood of vaccine virus shedding following the first dose of RV1, but no significant difference in RV1 vaccine virus shedding overall. The proportion of infants with RV1 vaccine virus shedding overall was similar in non-secretors (40%) and secretors (55%) and peak shedding level did not differ between the two groups. There was no difference in RV1 vaccine virus shedding between Lewis negative and Lewis positive infants at any timepoint. Stratifying secretor and non-secretor infants by Lewis negative phenotype did not significantly alter the observed lack of association between HBGA phenotype and RV1 vaccine virus shedding.

Preliminary findings presented at recent conferences by groups in Nicaragua and Burkina Faso have reported some association between secretor phenotype and vaccine virus shedding, however, to date, there are no published data in this field (Reyes et al., 2017).

These data confirm that non-secretor/Lewis negative infants do show evidence of vaccine virus replication, and therefore any resistance to G1P[8] infection, at least to the vaccine strain, is clearly relative rather than absolute. This might allow an adequate immune response, which will be tested and discussed further in Chapter 4. This might also suggest that non-secretor/Lewis negative infants, while protected from P[8] RV gastroenteritis, might remain susceptible to asymptomatic P[8] RV infection. This new hypothesis will be discussed further and tested in Chapter 5.

Relationship between demographic and socioeconomic factors and RV1 vaccine virus shedding

There was no evidence that HIV exposure was associated with reduced or increased vaccine virus shedding, although numbers of HIV exposed infants were small, and no infant was known to be HIV-infected at 6 weeks. This is consistent with previous studies which found no evidence of reduced vaccine take to rotavirus vaccines in HIV-infected or HIV exposed infants, and no evidence of prolonged vaccine virus shedding (Steele et al., 2011, Steele et al., 2009, Levin et al., 2017).

There was some evidence that poorer nutritional status was associated with reduced vaccine virus shedding. Both baseline weight for age and MUAC were positively associated with vaccine virus shedding. A 1cm increase in MUAC or 1 standard deviation increase in weight for age was associated with around a 20% increase in overall likelihood of RV1 vaccine virus shedding. This apparent effect on viral replication is consistent with recent evidence from Bangladesh, which suggested that malnutrition was associated with relative protection against natural rotavirus infection (Verkerke et al., 2016). The authors suggested that shortening of intestinal villae, due to malnutrition-associated environmental enteropathy, could be a barrier to rotavirus attachment and replication. However, in the cohort the association with nutritional parameters was modest, and only weight for age retained significance in multivariable analysis. Of note,

there is limited evidence that nutritional status affects vaccine immunogenicity or efficacy (see Chapter 1).

We explored the relationship between several markers of poverty and poor sanitation and RV1 vaccine virus shedding. The only significant association found was a positive association between RV1 vaccine virus shedding and maternal education. In univariable analysis, for every one-year increase in maternal education, there was a 5% increase in likelihood of RV1 vaccine virus shedding. The effect did not retain significance in multivariable analysis. Low maternal education is known to be associated with poverty and poor child health outcomes (Boyle et al., 2006). In Malawi, increasing maternal education level has been shown to be associated with decreased child mortality (Makate and Makate, 2016). In Malawi, primary education has been free for all since 1994. But the indirect and opportunity costs of schooling may mean in poorer families, children, particularly girls, may stay at home. Early marriage and teenage pregnancy also limit secondary education opportunities.

If low maternal education is a proxy for poverty, why were no other proxy markers of poverty or sanitation associated with lower vaccine virus shedding? The indicators chosen were based on previous studies in Malawi, but some of these were based on older data, and socioeconomic indicators are likely to change in significance over time. It may be chosen markers of poverty were too insensitive to stratify poverty within this very poor population. My data may be subject to floor effects, where low, lower and lowest levels of wealth and sanitation were not distinguished. Maternal education may be a more sensitive indicator.

It may also be that poverty is a significant predictor of poor rotavirus vaccine response, but that this occurs below a certain threshold, and in this uniformly poor population in one of the world's least developed countries, there was simply no higher income comparison group to demonstrate the effect.

Limitations

A high proportion of families declined to participate or withdrew consent before data collection began. This is not unexpected in an infant study with no direct benefit to families and multiple blood sampling points, particularly in this setting. Attrition during follow-up was around 21%,

which is also not unexpected in this setting where families are highly mobile due to family commitments in villages outside Blantyre. By definition, no data were collected on families who declined to participate. However, demographic characteristics of participating families were as expected based on census data from this population. Infants who withdrew or defaulted after data collection began were compared to those who completed the study and no significant demographic differences were found. In addition, the study population was highly similar to the randomly selected community controls recruited to the case:control study. The potential limitation of recruitment bias is acknowledged, but the evidence available suggests that any bias is minimal and that the study population is representative of the local population.

Logistical difficulties and infrequent stooling patterns (common in breast-fed infants) meant that not all infants had stool samples available for every time point. This limited the sample size slightly, since only infants with sufficient stool samples per dosing period were included, and could lead to an underestimation of vaccine virus shedding rates. In contrast, the highly sensitive RT-PCR methods used could over-estimate shedding rates, although this potential limitation was reduced by the inclusion of VP6 qRT-PCR as a confirmatory assay, and the relatively conservative definition of shedding used where two positive samples were required to confirm vaccine virus shedding. On balance, estimation of vaccine virus shedding rates in this population are likely to be as robust as logistically possible.

Sample size in OPV analysis was unavoidably limited by the global switch to bivalent OPV in late April 2016. As a result, OPV results should be interpreted with some caution.

HBGA phenotyping showed a higher than expected proportion of infants who were positive for both Lewis a and Lewis b antigen. Most of these infants were phenotypic secretors, Le^{a+b+} “partial secretor” phenotype is much more common in young infants (Ameno et al., 2001). However, eleven Le^{a+b+} infants were FUT2-genotype confirmed non-secretors. In these infants it is likely that Lewis b positivity was secondary to cross-reactivity between Lewis a and b antigens in the ELISA assay. Lower specificity in the Lewis ELISA could also explain the unexpected finding of Le^{a-b+} phenotype in three genotype confirmed non-secretor infants. One further Le^{a-b+} infant was not genotyped but was found to be a phenotypic secretor at time of second immunisation.

Similarly, three infants initially classed as phenotypic non-secretors were found to be heterozygous secretors by FUT2 genotyping, and two of these infants were phenotypic secretors by the time of second immunisation. This pattern could relate to the developmental expression of FUT2/FUT3 genes where the Lewis antigens are expressed first, however the possibility of a false negative ABH ELISA on first testing cannot be excluded. It should be noted, that in most infants confirmatory genotyping was generally consistent with ABH non-secretor phenotyping results, suggesting false negative results were uncommon. It should also be noted that given the high sensitivity of both Lewis assays, the determination of Lewis negative (Le^{a-b-}) phenotype in infants can be considered reasonably robust.

Gene sequencing may have offered greater specificity in determining Lewis and secretor genotype, but was not feasible in this setting due to logistical and ethical constraints.

Summary

The data collected on vaccine virus shedding has allowed us to better describe shedding patterns in this Malawian population. Passively acquired RV-specific IgG and weight for age at time of first immunisation have emerged as the best predictors of vaccine virus shedding, with more modest effects seen for OPV vaccine virus shedding, other parameters of nutritional status and maternal education. Vaccine virus replication is an important indicator of vaccine response, but not a direct determinant of protection. In the next chapter we will explore how shedding relates to immune response.

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4 PREDICTORS OF RV1 IMMUNOGENICITY

4.1 INTRODUCTION

Although there is no proven correlate of protection for rotavirus vaccines, there is consensus that serum rotavirus-specific IgA (RV-specific IgA) response is the closest known surrogate of protection and best available measure of vaccine immunogenicity (Angel et al., 2014). At a population level, in parallel to vaccine efficacy, post-immunisation RV-specific IgA responses are much lower in low income compared to high income countries (Patel et al., 2013). For example, in pooled estimates from vaccine trials, post-immunisation RV-specific IgA in Malawi was 52 (26-102) U/mL compared to 338 (266-429) U/mL in the US and Europe (Patel et al., 2013). Seroconversion rates follow a similar gradient, with rates in low income settings of around 50% compared to almost 90% in high income settings (Patel et al., 2013). In seeking to establish factors contributing to lower vaccine efficacy in low-income, high mortality settings, it is therefore reasonable to first determine factors associated with reduced RV-specific IgA response.

In natural rotavirus infection, virus replication is required to stimulate a mucosal immune response, and live oral rotavirus vaccines are assumed to act by similar mechanisms (Arnold et al., 2013, Clarke and Desselberger, 2015). RV-specific IgA response may therefore be reduced indirectly by any factor which reduces vaccine virus replication, directly by factors which diminish the host mucosal immune response, or by a combined mechanism by factors which affect both (Figure 4-1). In Chapter 3, factors which may reduce RV-specific IgA indirectly through reduced vaccine virus replication were discussed, with passively-acquired maternal antibody and HBGA phenotype identified as factors most significantly associated with reduced vaccine virus shedding. Mechanistic concepts predict that vaccine viral replication will determine humoral immune response, but this is likely to be a complex, non-linear relationship. For example, threshold effects might require a minimum level of viral replication to stimulate host-response, and ceiling effects might limit this response above a certain level. There may also be different relationships between first and subsequent vaccine doses. Robust vaccine

virus replication in the naïve host at 1st dose which stimulates an effective humoral response may result in reduced vaccine virus replication with subsequent doses. In this chapter, we will examine whether factors proposed to affect shedding in Chapter 3 also significantly reduce RV-specific IgA response.

Transplacental transfer of maternal IgG occurs primarily in the 3rd trimester. While protecting the infant from infection in early life, passively-acquired IgG has been demonstrated to inhibit the infant's own immune response to various vaccines (Siegrist, 2003). The effect is not universal, and may vary in extent dependent on the specific vaccine antigen and vaccine antigen/maternal antibody ratio (Jones et al., 2014). Inhibition by maternal IgG is specific to B-cell responses and T-cell responses appear unimpaired (Gans et al., 1999, Crowe et al., 2001). Several mechanisms of inhibition have been proposed based on clinical observations and mechanistic studies. These include direct neutralization of replicating vaccine virus; epitope masking by passively-acquired IgG preventing B cell priming; effective uptake and elimination of maternal antibody-antigen complexes by infant antigen presenting cells; and inhibition of infant B cell activation via Fcγ-receptor mediated signalling (Niewiesk, 2014). The relative contribution of each of these mechanisms is subject to debate (Kim et al., 2011, Siegrist, 2003). Whichever model is correct, the outcome is passively-acquired maternal IgG-mediated suppression of the infant B cell response. These proposed mechanisms of inhibition have largely focused on the systemic humoral immune response to parenteral vaccines. Understanding of mechanisms by which maternal IgG might inhibit mucosal immune response to live oral vaccines is more limited, but similar B-cell mediated mechanisms of inhibition to those described above in blood could occur in the sub-mucosa. In addition, as described in chapter 3, transcytosis of maternal IgG into the intestinal lumen could directly block vaccine virus replication (Westerman et al., 2005). There is some evidence from animal studies that high maternal IgG can suppress mucosal immune response to rotavirus infection. Parreno et al. (1999) showed that both serum and intestinal RV-specific IgA responses to rotavirus infection were reduced in gnotobiotic pigs passively-immunised with high-titre maternal antibody.

In Malawi, a high burden of rotavirus could result in high maternal RV-specific IgG and consequently greater inhibition of infant rotavirus vaccine response. However, preterm delivery

and maternal co-morbidities such as HIV and malaria are common in Malawi, and could reduce transplacental transfer (Palmeira et al., 2012).

Concurrent OPV immunisation could inhibit RV-specific IgA indirectly through competitive inhibition on viral replication (Wang et al., 2012), but also through direct effects on the mucosal immune response. Interference between live viral vaccine strains with inhibition of vaccine response has been well documented for multivalent vaccines containing sub-strains of the same virus, for example trivalent OPV (Patriarca et al., 1991) and tetravalent dengue vaccine (Anderson et al., 2011), and between different viruses, for example suppression of rubella response in early combined measles-mumps-rubella vaccines (Berger et al., 1988, Berger and Just, 1988). Mechanisms of interference are not well understood, but could occur through direct competitive inhibition of viral replication or through competition for available resources such as antigen-presenting cells, limiting T Helper activation and therefore B cell response. Animal studies have provided proof of principle of bidirectional effects on both innate and cell-mediated response during simultaneous co-infection with the unrelated viruses lymphocytic choriomeningitis virus, Pichinde and ectromelia virus (McAfee et al., 2015, Kenney et al., 2015).

Potential immune-interactions of rotavirus vaccine and OPV have been little explored. Both rotavirus and polioviruses have strategies to down-regulate the innate anti-viral immune response, primarily by downregulation or inhibition of innate signalling pathways (Liu et al., 2009, Lei et al., 2016). Interaction of such strategies could potentially inhibit innate pathways to immune cell recruitment and limit the adaptive response. Wang et al. (2012), in an invitro study of co-infection of cells by rotavirus alone or in combination with enteroviruses, showed elevated expression of seven cytokine genes in dual compared to single infection. The overall effect of these complex interactions is unpredictable, and may depend on both which vaccine virus epitopes are immunodominant and prior priming (Kedl et al., 2003). Priming may be key to the inhibitory effect of OPV on rotaviruses – most infants receive a birth dose of OPV. At 6 weeks, infants without prior natural infection will be naïve to rotavirus, but already primed to OPV. In addition, OPV responses do not appear to be significantly inhibited by passively-acquired maternal antibody (Bavdekar et al., 1999). This could potentially offer OPV a competitive advantage over rotavirus.

The determinants of the development of infant innate and adaptive immunity are diverse. Genetics, early life antigenic exposure, priming effects from maternal immunity, the evolving microbiome, fetal and neonatal nutrition all play a role (Kampmann and Jones, 2015, Cunningham-Rundles et al., 2009). Low birth weight, with or without prematurity, may impact on cellular immunity and vaccine response in early life (Saha et al., 1983, Das et al., 1998). HIV infection and exposure may both impact early immune development and vaccine response and may directly affect gut health (Jones et al., 2011). Both low birth weight and HIV infected and HIV exposed, uninfected infants are at higher risk of poor growth and malnutrition (Rosala-Hallas et al., 2017, Isanaka et al., 2009, Akombi et al., 2017). Malnutrition, particularly micronutrient deficiency, can adversely affect both immune development and gut epithelial integrity. Poverty, poor sanitation, malnutrition and high burden of enteric infection all contribute to the development of poor gut health which in turn is associated with chronic immune activation and mucosal immune dysfunction (Prendergast and Kelly, 2012). This thesis will not attempt to address all these complexities. However, analysis was made of the association between RV-specific IgA response and the following demographic factors: low birth weight, HIV exposure, nutritional status, and socioeconomic markers of poverty/sanitation.

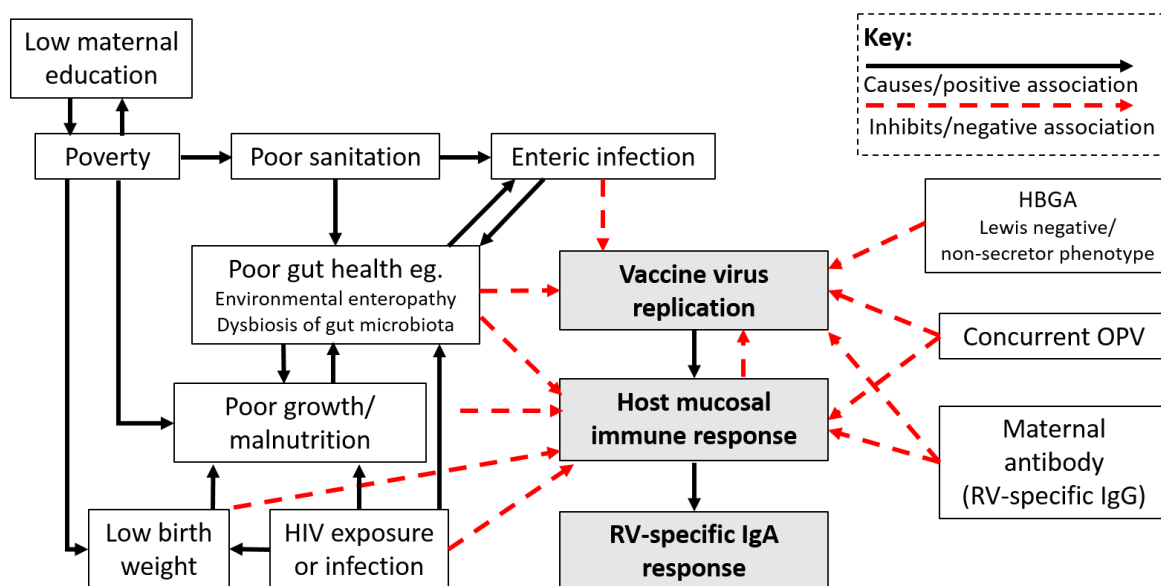


Figure 4-1: Proposed factors contributing to reduced RV-specific IgA response

4.2 AIMS

My first aim was to describe the immunogenicity of this schedule in this setting, and to confirm the association between vaccine virus shedding (as a proxy for vaccine virus replication) and RV-specific IgA response.

I then aimed to determine the relationship between RV-specific IgA response to RV1 vaccination and three potentially modifiable factors: passively-acquired maternal antibody; post-immunisation polio vaccine virus shedding; and HBGA phenotype.

Finally, as a secondary aim I explored the relationship between RV-specific IgA response and low birth weight, HIV exposure, nutritional status and proxies of poverty and poor sanitation.

I tested the following hypotheses:

4.2.1 Hypotheses

Primary hypotheses

1. RV1 vaccine virus shedding

- a. The presence of RV1 vaccine virus shedding will predict RV-specific IgA seroconversion.
- b. Quantitative RV1 vaccine virus shedding will be correlated with quantitative RV-specific IgA response.

2. Passively-acquired maternal antibody (RV-specific IgG)

Higher levels of passively-acquired maternal rotavirus antibody (RV-specific IgG) at time of 1st RV1 immunisation will be associated with reduced RV-specific IgA response.

3. Oral Polio Vaccine response

- a. OPV shedding at time of rotavirus immunisation will be associated with reduced RV-specific IgA response.
- b. Protective OPV neutralizing antibody response will be associated with reduced RV-specific IgA response.

4. HBGA phenotype

- a. Lewis negative HBGA phenotype will be associated with reduced RV-specific IgA response.
- b. Non-secretor HBGA phenotype will be associated with reduced RV-specific IgA response.

Secondary Hypotheses

5. Other host factors

- a. Low-birth weight infants will have reduced RV-specific IgA response.
- b. HIV exposed infants will have reduced RV-specific IgA response.
- c. Infants with poor nutritional status at time of immunisation will have reduced RV-specific IgA response.

5. Socioeconomic and Sanitation factors

- a. Predictors of poverty will be associated with reduced RV-specific IgA response.
- b. Predictors of poor sanitation will be associated with reduced RV-specific IgA response.

4.3 METHODS

Detailed methods are given in Chapter 2. In brief, this was a longitudinal cohort study. Infants were recruited from Zingwangwa Health Centre, Blantyre, Malawi, prior to 1st RV1 immunisation at around 6 weeks of age. Infants with known conditions causing immunosuppression (other than HIV) and those not expected to be resident in Blantyre throughout the study period were excluded.

Social and demographic data were collected by structured interview. Infant characteristics, including low birth-weight and gender, which could be associated with reduced immune host response were recorded. Key socio-economic data which could potentially be associated with reduced immunogenicity were included for analysis. Definitions are provided in Chapter 2.6.

Infant and maternal HIV status was determined by verbal report and from hand-held health records as detailed in Chapter 2.6. HIV-exposure was defined as any infant of an HIV-infected mother. HIV-infection in infants was confirmed by DNA PCR test at 6 weeks. HIV DNA PCR tests were performed by Ministry of Health clinical staff and not as part of the study. Results were reported by families to the study team when available.

Nutritional status was determined by length, weight and mid-upper arm circumference measurement at time of recruitment, and at each follow-up visit, and compared to WHO age-determined z scores.

4.3.1 Laboratory methods

Laboratory methods are described in detail in Chapter 2.

The primary outcome measure was RV-specific IgA seroconversion. RV-specific IgA was determined by sandwich ELISA at baseline prior to 1st RV1 dose and two weeks following the 2nd RV1 dose. Seroconversion was defined as a change from seronegative (rotavirus-specific IgA < 20 U/mL) at baseline to seropositive (RV-specific IgA > 20 U/mL) post-immunisation, or at least a four-fold rise in RV-specific IgA concentration post immunisation in infants seropositive at baseline. Secondary immunogenicity outcome measures were seropositivity and proposed protective response. Seropositivity was defined as a post-immunisation RV-specific IgA concentration of > 20 U/mL, in infants seronegative (RV-specific IgA < 20 U/mL) at baseline. Proposed protective response was defined as post-immunisation RV-specific IgA > 90 U/mL in infants who were seronegative at baseline.

The primary predictive measures were RV1 and OPV vaccine virus shedding, poliovirus 1 and poliovirus 3 neutralizing antibody response, RV-specific IgG, and HBGA phenotype. Wild-type rotavirus infection at time of immunisation was included as an exploratory measure.

Stool samples for RV1, OPV and wild-type rotavirus shedding were taken prior to each RV1 dose and on alternate days from days 2-10 post immunisation. A minimum of two post-immunisation samples were required for infants to be included in the shedding analysis. RV1 shedding was determined by vaccine-specific NSP2 RT-PCR and confirmed by VP6 qRT-PCR. RV1 vaccine virus

shedding was defined as two or more positive NSP2 positive, VP6 positive samples from days 4-10 post immunisation. Level of shedding was quantified using peak shedding, defined as the reciprocal of the minimum NSP2 Ct value of all positive samples within each dose period.

OPV virus shedding was determined by multiplex RT-PCR for Sabin sub-types 1-3. OPV virus shedding was defined as any two positive samples of any Sabin sub-type from days 4-10 post immunisation.

RV-specific IgG at baseline was determined by sandwich ELISA.

Poliovirus-specific neutralizing antibody responses to serotypes 1 and 3 were determined prior to routine immunisation around 6 weeks, and two weeks following the subsequent dose.

Poliovirus-specific neutralizing antibody responses to serotype 2 could not be tested due to the ban on use of live Sabin 2 viruses in laboratories following the global switch to bivalent OPV in April 2016. Neutralizing antibody titres were determined using a micro-neutralization assay. Titres greater than 8 were considered protective (seropositive)(Sutter et al., 1995). This definition and methodology used are consistent with WHO agreed international standards for polio serology. Poliovirus-specific seroconversion between six and twelve weeks was defined as either a change from seronegative at six weeks to seropositive at twelve weeks, or greater than a four-fold rise in titres between six and twelve weeks.

HBGA phenotype at baseline was determined by salivary ELISA for A, B, H and Lewis a and b antigens. Infants who were A, B or H positive were classified as secretors. Infants who were negative for ABH were confirmed as non-secretors by Lectin ELISA and by FUT2 genotyping. Infants who were positive for either Lewis a or Lewis b antigen were classified as Lewis positive, and those negative for both Lewis antigens as Lewis negative. Final phenotype was determined by two ELISA tests (ABH or Lectin) confirming the same phenotype. HBGA phenotype at time of 2nd dose was determined only for infants who were non-secretors at 1st dose. The relationship between immunogenicity and HBGA phenotype was based on HBGA phenotype at baseline, but with sensitivity analyses based on HBGA phenotype at 2nd dose and HBGA genotype. Analysis was made by unstratified and stratified Lewis/secretor HBGA phenotype.

4.3.2 Statistical analysis

Summary statistics were described as mean and 95% confidence interval for normally distributed continuous variables, median and intra-quartile range for non-normally distributed continuous variables and proportion and 95% confidence interval of proportion for categorical variables. Geometric mean concentration of RV-specific IgA was calculated as this is standard practice in vaccine response studies. To explore both linear and non-linear effects, antibody and shedding variables were analysed as both continuous variables and categorized in quantiles to create ordinal variables. Correlations between non-parametric continuous variables were made by Spearman's correlation. Relationship between categorical outcomes and predictor variables was made by binomial logistic regression. Multivariable models were created using binomial logistic regression, or by Poisson regression with robust errors where there was failure of the binomial logistic model to converge. The model was built using a manual stepwise backward elimination approach. All variables significant in univariable analysis to $p < 0.1$ were initially included in the multivariable model. Variables with the highest p value were removed sequentially until only variables with $p < 0.1$ remained. The Benjamini-Hochberg procedure was applied to adjust for multiple comparisons, with a 25% false discovery rate (Benjamini and Hochberg, 1995). All statistical analysis was made using StataIC version 13.1 (StataCorp, US).

4.4 RESULTS

4.4.1 Recruitment

Recruitment screening for the cohort study began in April 2015 with the first participant commencing the study in May 2015 (Figure 4-2).

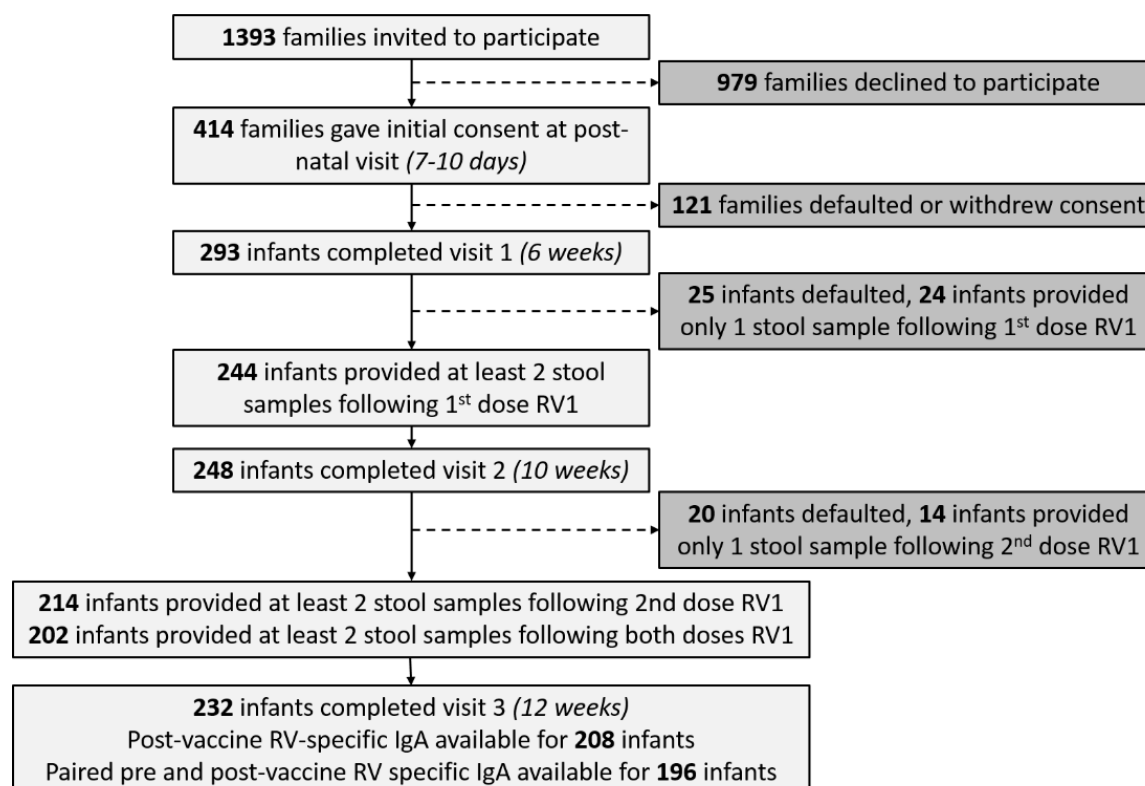


Figure 4-2: Recruitment flow diagram for immunogenicity cohort

Recruitment was completed in August 2016. Attrition is discussed in detail in Chapter 3. Of infants who completed data collection at the first visit at 6 weeks, 232/296 (78%) infants completed the full follow-up to 12 weeks. Of these, 196/232 (84%) had paired blood samples for RV-specific IgA pre- and post-immunisation. Of these, 165/232 (71%) infants had complete vaccine virus shedding data for both dose periods and paired serology.

4.4.2 Demographics of the immunogenicity cohort

Demographics of 196 infants included for immunogenicity analysis are summarized in Table 4-1. Population demographics were similar to those included in the shedding analysis (Chapter 3). Vaccination was highly timely, 192/196 (98%, 95-99%) infants received the first RV1 dose at 6±2 weeks of age and 186/196 (95%, 91-97%) received the second RV1 dose at 10±2 weeks of age. Prevalence of low-birth weight (12%, 8-17%) and HIV exposure (15%, 11-21%) were high, as expected for this setting (Chapter 2.6). Of 29 HIV exposed infants, 14 had an HIV DNA PCR result known by the end of follow-up – all were HIV uninfected. All HIV exposed infants received six weeks Nevirapine anti-retroviral prophylaxis.

Almost all infants were exclusively breast-fed (Table 4-1). At 6 weeks old, very few infants were severely underweight (weight for age z score <3SD, 5/196, 3% ,1-6%) or severely wasted (weight for length z score <3SD, 4/190, 2%, 0.7-6%). Median anthropometric z scores were close to zero, reflecting expected WHO age distribution (Table 4-1). As expected for this low-income peri-urban setting, there was a high proportion of families with indicators of poor sanitation, poverty and food insecurity (Chapter 2.6).

Table 4-1: Demographic characteristics of immunogenicity cohort

Characteristic	Immunogenicity Cohort
<u>Timing of vaccine</u>	
Median age in weeks at 1 st RV1 dose (IQR)	6.1 (6.0-6.3)
Median age in weeks at 2 nd RV1 dose (IQR)	10.6 (10.4-10.9)
Median time between RV1 doses, weeks, (IQR)	4.4 (4.3-4.4)
<u>Infant characteristics</u>	
Male	94 (48%, 41-55%)
Exclusively breast-fed	195 (99%, 96-100%)
HIV-exposed	29/195 (15%, 11-21%)
Low birth weight (<2.5kg)	22/190 (12%, 8-17%)
<u>Nutritional status</u>	
Median weight for age z-score at 1 st RV1 dose (IQR)	-0.46 (-1.2-0.31)
Median length for age z-score at 1 st RV1 dose (IQR)	-0.70 (-1.88- -0.02) n=190
Median weight for length z-score at 1 st RV1 dose (IQR)	0.39 (-0.52-1.35) n=190
Median MUAC at 1 st RV1 dose, cm, (IQR)	12.4 (11.5-13)
<u>Sanitation and socioeconomic predictors</u>	
Median household size (IQR)	5 (4-6)
Non-piped water source (borehole or well)	23 (12%, 8-17%)
Time taken to access water	
<5minutes	69/195 (35%, 29-42%)
5-30 minutes	103/195 (53%, 46-60%)
>30 minutes	23/195 (12%, 8-17%)
Shared toilet	78 (77%, 70-82%)
Electricity at home	98 (50%, 43-57%)
One or more household member with salary	155 (79%, 73-84%)
Household food insecurity	64 (33%, 26-40%)
Median age of head of household, years (IQR)	32 (28-38.5)
Median years of maternal education (IQR)	10 (8-12)

Categorical variables are reported as number, (proportion, 95%CI of proportion). Denominator n= 196 unless otherwise stated.

4.4.3 IgA response following RV1 immunisation at 6 and 10 weeks

Six percent of infants (11/196 (6%, 3-10%)) were seropositive (RV-specific IgA >20U/mL) prior to RV1 immunisation at 6 weeks. Six of these infants provided a pre-immunisation stool sample, of whom 5 were shedding low levels of wild-type rotavirus.

Post-immunisation samples were collected a median of 15 (IQR 14-16) days following the 2nd dose of RV1, at a median age of 12.7 (IQR 12.4-13.3) weeks. Just over half (107/196 (55%, 48-61%)) of the cohort had RV-specific IgA above the minimum detectable level post-immunisation. The geometric mean concentration post-immunisation overall (including infants with undetectable IgA assigned a value of 1 U/mL) was 5.7 (95%CI 4.3-7.5) U/mL. In those infants with a detectable RV-specific IgA response, the geometric mean concentration was 25.7 (95%CI 18.5-35.8) U/mL. Seroconversion (a change from seronegative to seropositive, or at least a four-fold rise in RV-specific IgA post immunisation in those seropositive at baseline) was found in 47/196 (24%, 18-31%) infants. Excluding infants who were seropositive at baseline, 45/185 (24%, 19-31%) infants were seropositive (RV-specific IgA >20U/mL) post-immunisation, and 23/185 (12%, 8-18%) had RV-specific IgA above the proposed protective level of 90U/mL.

4.4.4 RV-specific IgA response and RV1 vaccine virus shedding

Seroconversion was compared in 180 infants with complete shedding data for the 1st dose period, 175 infants with complete shedding data for the 2nd dose period, and 165 infants with complete shedding data over both dose periods. Seroconversion rates were associated with RV1 shedding post 1st dose: 25/54 (46%, 33-60%) seroconversion in those with shedding, compared to 19/126 (15%, 10-23%) in those without shedding after the 1st dose of RV1 vaccine. Infants with shedding after dose 1 were over three times as likely to seroconvert (RR 3.1 (95%CI 1.9-5.1), p<0.001). Although the trend was similar, no significant association was seen between seroconversion and shedding after the 2nd dose RV1: 17/59 (29%, 19-42%) seroconversion in those with shedding, compared to 23/116 (20%, 13-28%) seroconversion in those without shedding after the 2nd dose of RV1 vaccine (RR 1.5 (95% CI 0.84-2.5), p=0.18). Shedding after either dose was associated with seroconversion: 27/86 (31%, 22-42%) seroconversion in those with shedding, compared to 12/79 (15%, 9-25%) shedding in those without shedding after

either RV1 vaccine dose. Infants with any RV1 shedding were over twice as likely to seroconvert (RR 2.1 (95%CI 1.1-3.8), $p=0.02$). Post vaccine RV-specific IgA response was positively correlated with peak shedding (determined by the reciprocal of the minimum Ct value) after 1st dose of RV1: Spearman's R 0.46, $p<0.001$. A much weaker positive correlation was observed between post vaccine RV-specific IgA response and peak shedding (determined by the reciprocal of the minimum Ct value) after 2nd dose of RV1: Spearman's R 0.28, $p=0.04$.

To explore non-linear threshold effects, infants with shedding after each dose were categorized as “low shedding” if peak shedding was below the median for this population, and “high shedding” if peak shedding was above the median. Infants with high and low shedding were compared to infants with no shedding. Post vaccine RV-specific IgA response varied significantly by shedding group following 1st dose RV1, with higher shedding associated with a higher antibody response (Figure 4-3). Post-immunisation RV-specific IgA GMC in infants with no shedding was 3.73 (95%CI 2.7-5.1) U/mL compared to 8.9 (95%CI 3.8-21.2) U/mL in infants with low shedding and 39.2 (95%CI 21.9-70.3) U/mL in infants with high shedding (Kruskal Wallis test $p<0.001$). In binomial regression, infants with low level shedding were over twice as likely to seroconvert (RR 2.1 (95%CI 1.1-4.0), $p=0.03$) and infants with high shedding were 4.5 times as likely to seroconvert (RR 4.5 (95%CI 2.7-7.5), $p<0.001$) than infants with no shedding after 1st dose RV1.

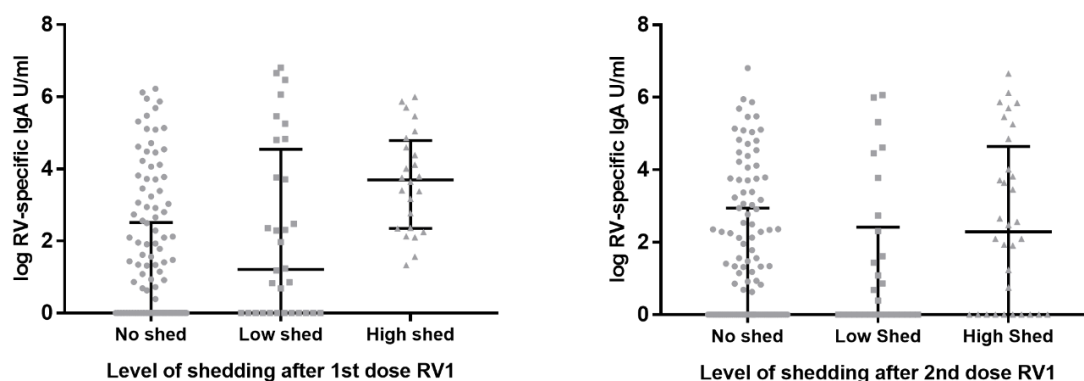


Figure 4-3: RV-specific IgA response by level of shedding after 1st dose of RV1

Post-immunisation RV-specific IgA is log-transformed for easier visualization. Undetectable RV-specific IgA was assigned an arbitrary value of 1 (log transformed=0). Error bars show median and intra-quartile range. No shed: fewer than 2 samples positive for RV1. Low shed: peak shedding value below the median. High shed: peak shedding value above the median.

A similar dose response relationship was not clearly seen when determining the relationship between RV-specific IgA response and level of shedding following 2nd dose of RV1 (Figure 4-3). Post-immunisation RV-specific IgA GMC in infants with no shedding following the 2nd dose was 4.6 (95%CI 3.3-6.5) U/mL compared to 4.0 (95%CI 1.9-8.5) U/mL in infants with low shedding and 13.0 (95%CI 5.7-29.6) U/mL in infants with high shedding (Kruskal Wallis test $p=0.04$).

In binomial regression, there was no significant association between infants with low level shedding following 2nd dose RV1 and seroconversion compared to infants with no shedding (RR 0.72 (95%CI 0.27-1.9), $p=0.51$). However, infants with high shedding following the 2nd dose RV1 were over twice as likely to seroconvert (RR 2.1 (95%CI 1.2-3.7), $p=0.008$) than infants with no shedding.

Infants with RV1 shedding only after the 2nd dose were less likely to seroconvert (seroconversion 6/38 (16%, 7-32%)) than those who shed only after the 1st dose (11/30, 37%, 21-56%) or after both doses (10/18, 56%, 31-78%), Fisher's exact $p=0.008$.

To determine the utility of shedding as a measure of vaccine take sensitivity and specificity were determined with seroconversion as the gold standard. RV1 shedding after either dose predicted seroconversion with a sensitivity of 69% (52-83%) and specificity of 53% (44-62%). RV1 shedding after the first dose had a higher specificity (79%, 11-85%) but lower sensitivity (56%, 41-72%).

4.4.5 Passively-acquired maternal antibody and RV-specific IgA response

Geometric mean concentration of RV-specific IgG at 6 weeks was lower in infants who seroconverted (2847 U/mL) compared to those who did not seroconvert (3968 U/mL), but the difference was not significant (t-test $p=0.07$, IgG log-transformed for normal distribution).

There was a weak negative correlation between RV-specific IgG at 6 weeks, and RV-specific IgA response following RV1 immunisation: Spearman's $R -0.20$, $p=0.005$.

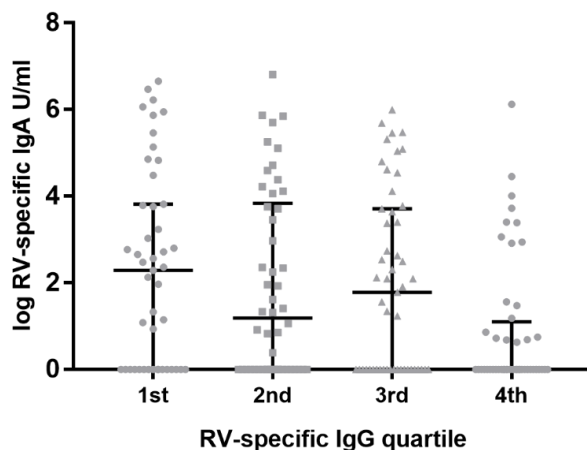


Figure 4-4: RV-specific IgA response by IgG quartile

Post-immunisation RV-specific IgA is log-transformed for easier visualization. Undetectable RV-specific IgA was assigned an arbitrary value of 1 (log-transformed value= 0). Error bars show median and intra-quartile range. RV-specific IgG measured at 6 weeks and divided into quartiles (<25th, 25th-50th, 50th-75th, >75th centile).

In binomial regression with log-transformed IgG as a continuous variable, RV-specific IgG a was not significantly associated with seroconversion: RR 0.82 (95%CI 0.65-1.0), $p=0.008$).

To explore non-linear threshold effects, infants were categorized by RV-specific IgG quartile. The distribution of RV-specific IgA was similar between infants with RV-specific IgG in the first 3 quartiles (Figure 4-4). However, infants with RV-specific IgG in the highest quartile had significantly lower RV-specific IgA response (GMC 2.42 (95%CI 1.6-3.8) U/mL than those with RV-specific IgG in the lowest quartile (GMC 10.1 (95%CI 5.2-19.6) U/mL (Mann-Whitney $p=0.002$))

The relationship between seroconversion and RV-specific IgG quartile was determined by binomial regression, with RV-specific IgG 1st quartile as the reference category. There was no significant decrease in likelihood of seroconversion between infants with RV-specific IgG in the 2nd (RR 0.94 (95%CI 0.50-1.8) $p=0.85$), and 3rd (0.86 (95%CI 0.45-1.6) $p=0.64$) quartile range, compared to those in the 1st quartile range. However, in the highest RV-specific IgG quartile, infants were was weak evidence that infants were less likely to seroconvert compared to those in the lowest quartile (RR 0.42 (95%CI 0.18-0.99), $p=0.05$).

4.4.6 Oral polio vaccine response and RV-specific IgA response

OPV shedding in the 1st dose period, was negatively associated with seroconversion (Table 4-2): 24/104 (23%, 16-32%) infants with OPV shedding in the 1st dose period seroconverted compared to 9/18 (50%, 26-74%) with no OPV shedding (Fisher's exact $p=0.02$). A similar trend was observed for OPV shedding in the 2nd dose period seroconversion: 15/62 (24%, 15-37%) infants with OPV shedding in the 2nd dose period seroconverted, compared to 14/41 (34%, 21-50%) infants with no OPV shedding, but this difference was not significant ($\chi^2 p=0.27$). In binomial logistic regression, OPV shedding in the 1st dose period was associated with a 64% decrease in the likelihood of seroconversion. There was no association between OPV shedding by Sabin sub-type in either dose period, but numbers of subgroups were small.

Table 4-2: OPV shedding and RV1 seroconversion

OPV Shedding Pattern	Rotavirus vaccine seroconversion RR (95%CI)
OPV shedding 1st dose period n=122	0.46 (0.26-0.82) $p<0.01$
Any SAB1 combination (1 st dose period)	0.89 (0.48-1.7) $p=0.70$
Any SAB2 combination (1 st dose period)	0.55 (0.29-1.0) $p=0.07$
Any SAB3 combination (1 st dose period)	0.86 (0.48-1.5) $p=0.62$
OPV shedding 2nd dose period n=103	0.71(0.38-1.3) $p=0.27$
Any SAB1 combination (2 nd dose period)	0.81(0.39-1.7) $p=0.58$
Any SAB2 combination (2 nd dose period)	0.98 (0.43-2.2) $p=0.96$
Any SAB3 combination (2 nd dose period)	0.82(0.43-1.6) $p=0.54$

There were insufficient numbers of infants with no OPV shedding following either dose to determine the association with rotavirus vaccine seroconversion by logistic regression. 4/4 (100%, 97-100%) infants with no OPV shedding seroconverted compared to 38/91 (44%, 33-54%) infants with OPV shedding after either dose (Fisher's exact $p<0.01$).

Infants receiving three doses of trivalent OPV, including a birth dose, were included for poliovirus-specific neutralizing antibody analysis. The proportion of these infants with protective poliovirus-specific neutralizing titres prior to 1st rotavirus dose at 6 weeks was 58/85

(68%, 57-71%) for serotype 1 and 26/85 (31%, 22-41%) for serotype 3. The majority of infants attained protective poliovirus-specific neutralizing antibody titres by 12 weeks: 78/85 (92%, 84-96%) for serotype 1 and 71/85 (84%, 74%-90%) for serotype 3. Between 6 and 12 weeks, 37/85 (44%, 33-54%) infants seroconverted to serotype 1 and 51/85 (60%, 49-70%) infants seroconverted to serotype 3.

A protective poliovirus-specific OPV neutralizing response for serotype 1, at either 6 or 12 weeks, was not associated with rotavirus vaccine seroconversion (Table 4-3). Serotype 1 poliovirus-specific seroconversion between 6 and 12 weeks was not associated with rotavirus vaccine seroconversion.

A protective poliovirus-specific OPV neutralizing response for serotype 3 at 6 weeks, was not associated with rotavirus vaccine seroconversion. However, a protective titre for serotype 3 at 12 weeks was strongly positively associated with rotavirus seroconversion: 21/71 (30%, 20-41%) of infants with protective poliovirus specific neutralizing antibody response for serotype 3 at 12 weeks also seroconverted to rotavirus vaccine. In comparison, of the 14 infants who failed to attain protective poliovirus serotype 3 neutralizing antibody response none seroconverted to rotavirus vaccine. There was no association between serotype 3 poliovirus-specific between 6 and 12 weeks and rotavirus vaccine seroconversion.

Table 4-3: Poliovirus-specific neutralizing antibody response and RV1 seroconversion

Poliovirus-specific neutralizing antibody response	Rotavirus vaccine seroconversion RR (95%CI)
Protective titres at 6 weeks	
Poliovirus 1	1.2 (0.51-2.7) p=0.72
Poliovirus 3	1.4 (0.66-3.0) p=0.38
Protective titres at 12 weeks	
Poliovirus 1	1.8 (0.28-11.5) p=0.54
Poliovirus 3	∞ (N/A)*
Seroconversion	
Poliovirus 1	0.80 (0.37-1.7) p=0.57
Poliovirus 3	1.1 (0.50-2.3) p=0.84

*unable to calculate risk ratio as 0/14 infants without protective poliovirus 3 titres attained RV1 seroconversion (see text).

There was a weak positive correlation between log-transformed polio-specific neutralizing antibody titres to serotype 3 and rotavirus-specific IgA at 12 weeks: Spearman's rho 0.23, $p=0.04$. There was no correlation between log-transformed polio-specific neutralizing antibody titres to serotype 3 at 6 weeks and rotavirus-specific IgA at 12 weeks: Spearman's rho 0.13, $p=0.24$. There was no correlation between serotype 1 titres at 6 or 12 weeks and rotavirus-specific IgA at 12 weeks: Spearman's rho 0.05, $p=0.68$ and 0.17, $p=0.13$, respectively.

4.4.7 HBGA phenotype and RV-specific IgA response

HBGA Lewis and secretor phenotype was determined for all 196 infants with paired seroconversion data. The distribution of HBGA phenotype for this study population is shown in Table 4-4.

Table 4-4 Distribution of secretor/ Lewis negative phenotype in seroconversion cohort

	Lewis positive	Lewis negative	Total
Non-secretor	29	16	45 (23%)
Secretor	120	31	151(77%)
Total	149 (76%)	47(24%)	196

Of the infants who were phenotypic non-secretors at 6 weeks old, 14/45 had insufficient whole blood samples available for FUT2 genotyping. Four of these infants were phenotypic secretors at 10 weeks old. Of the 31 phenotypic non-secretors with FUT2 genotyping data, 29 were confirmed as non-secretor genotype. Two infants were confirmed as heterozygous secretors, one of these infants had a secretor salivary phenotype at 10 weeks old and one remained a phenotypic non-secretor.

The proportion of infants with saliva positive for both Le^a and Le^b was high: 81/196 (41%, 35-48%) were Le^{a+b} . Most of these infants were phenotypic secretors: 70/151 (46%, 38-54%) secretors and 11/45 (24%, 14-40%) non-secretors were Le^{a+b+} . All eleven Le^{a+b+} non-secretor infants were confirmed as non-secretors by FUT2 genotyping. A further 13/45(29%, 17-44%) non-secretor infants were Le^{a+b-} and 16/45(36%, 23-51%) were Lewis negative (Le^{a-b-}). Unexpectedly, 4/45 (9%) phenotypic non-secretors were Le^{a-b+} . Three of these infants were

confirmed as non-secretors by FUT2 genotyping. One had no blood available for genotyping, but had secretor salivary phenotype at 10 weeks old.

Likelihood of seroconversion by HBGA Lewis and secretor phenotype was determined by binomial logistic regression. There was no significant association between secretor and Lewis phenotype and any measure of RV-specific IgA response (Table 4-5). Seroconversion rates by HBGA phenotype were similar: 6/45 (13%, 6-27%) non-secretors seroconverted, compared to 41/151 (27%, 21-35%) secretors (Fisher's exact test $p=0.07$); and 12/47 (26%, 15-40%) Lewis negative infants seroconverted compared to 35/149 (23%, 17-31%) Lewis positive infants (Fisher's exact test $p=0.84$).

Table 4-5: Risk ratio of seroconversion by HBGA phenotype

	Non-secretor RR (95%CI)	Lewis negative RR (95%CI)
Seroconversion <i>n</i> =47/196	0.49 (0.22-1.1) $p=0.08$ <i>n</i> =6/45	1.1 (0.61-1.9) $p=0.77$ <i>n</i> =12/45
Post IgA>20U/mL <i>n</i> =45/185*	0.52 (0.24-1.2) $p=0.11$ <i>n</i> =6/42	1.1 (0.60-1.9) $p=0.82$ <i>n</i> =12/47
Post IgA>90U/mL* <i>n</i> =23/185	0.72 (0.26-2.0) $p=0.52$ <i>n</i> =4/42	0.82 (0.32-2.1) $p=0.67$ <i>n</i> =5/47

**excludes infants seropositive at baseline*

In a further analysis stratifying secretor by Lewis phenotype, no significant association was found between seroconversion and any combination phenotype.

Table 4-6 Risk ratio of seroconversion stratified by secretor/Lewis negative phenotype

HBGA phenotype	Seroconversion <i>n</i> , %, RR (95%CI)
<u>Non-secretor</u>	<i>n</i> =6/45 (13%) 0.5 (0.2-1.1) $p=0.08$
<i>Lewis negative</i>	<i>n</i> =2/16 (13%) 0.9 (0.2-4.4) $p=0.90$
<i>Lewis positive</i>	<i>n</i> =4/29 (14%) 1.1 (0.2-5.4) $p=0.90$
<u>Secretor</u>	<i>n</i> =41/151 (27%) 2.0 (0.9-4.5) $p=0.08$
<i>Lewis negative</i>	<i>n</i> =10/31 (32%) 1.2(0.7-2.3) $p=0.46$
<i>Lewis positive</i>	<i>n</i> =31/120 (26%) 0.8 (0.4-1.4) $p=0.46$

Using RV-specific IgA as a continuous variable, there was no significant difference in the distribution of post-immunisation RV-specific IgA between secretors and non-secretors (Mann-Whitney test, $p=0.70$), or between Lewis positive and negative infants (Mann-Whitney test, $p=0.89$).

Additional analysis

Seroconversion was compared by Le^{a+b-} phenotype. There was no difference in RV1 seroconversion in Le^{a+b-} infants compared to infants with other Lewis phenotype: 4/21 (19%, 7-43%) with seroconversion compared to 43/175 (25%, 19-32%) respectively (Fisher's exact $p=0.79$).

There was no difference in the proportion of 151 secretor infants with RV1 seroconversion by ABO phenotype (Table 4-7). There was no difference in RV1 seroconversion when type O infants were compared to infants with non-O type: 14/65 (22%, 13-34%) of type O infants seroconverted compared to 27/86 (31%, 22-42%) non-O type infants (χ^2 $p=0.18$).

Table 4-7: RV1 seroconversion by ABO type

ABO Type	O	A	B	AB	p value*
RV1 seroconversion	14/65, 22% (13-34%)	16/49, 33% (21-47%)	9/30, 30% (16-50%)	2/7 (29%) (4-78%)	0.56

*Fisher's exact test

4.4.8 Demographic and socio-economic factors

Timing of vaccination strictly followed the EPI schedule with little variation and no association between timing of vaccine and seroconversion was found.

There was no association between seroconversion and low birth weight: 5/22 (23%, 9-46%) low birth weight infants seroconverted, compared to 40/168 (24%, 18-31%) infants with normal birth weight (Fisher's exact $p=0.58$).

There was no association between seroconversion and HIV exposure: 3/29 (10%, 3-29%) HIV-exposed infants seroconverted, compared to 46/166 (26%, 20-33%) infants with no HIV exposure (Fisher's exact $p=0.10$).

No measure of nutritional status at baseline was associated with seroconversion.

No predictor of poor sanitation, over-crowding or poverty was associated with seroconversion.

Table 4-8: Risk ratio of seroconversion by infant and socioeconomic predictors

Characteristic ^a	OR	p ^b
<u>Timing of vaccination</u>		
Age in weeks at 1 st RV1 dose	1.4 (0.94-1.9)	0.10
Age in weeks at 2 nd RV1 dose	1.2 (0.8-1.7)	0.48
Time in weeks between RV1 doses	0.44 (0.15-1.3)	0.13
<u>Infant characteristics</u>		
Male	1.0 (0.63-1.7)	0.88
HIV-exposed	0.40 (0.13-1.2)	0.10
Low birth weight (<2.5kg)	0.95 (0.42-2.2)	0.91
<u>Nutritional status</u>		
Weight for age z-score (at baseline)	0.99 (0.78-1.2)	0.92
Length for age z-score (at baseline)	0.94 (0.78-1.1)	0.50
Weight for length z-score (at baseline)	1.1 (0.89-1.3)	0.53
MUAC (cm)	0.88 (0.70-1.1)	0.29
<u>Sanitation and socioeconomic predictors</u>		
Household size	0.93 (0.79-1.1)	0.40
Non-piped water source	0.33 (0.09-1.3)	0.11
Time taken to access water		
<5minutes	ref	
5-30 minutes	0.99 (0.58-1.7)	0.96
>30 minutes	0.75 (0.38-1.5)	0.41
Shared toilet	1.1 (0.61-2.1)	0.69
Electricity at home	1.0 (0.63-1.7)	0.87
One or more household members with salary	1.5 (0.73-3.1)	0.26
Household food insecurity	0.71 (0.39-1.3)	0.25
Age of head of household	1.0 (0.99-1.0)	0.32
Years of maternal education	1.1 (0.98-1.2)	0.14

a. Numbers given in Table 4-1

4.4.9 Multivariable model

All variables significant to $p < 0.10$ in bivariable analysis were considered for inclusion in a multivariable binomial regression model. Where variables were collinear, for example overall and sub-groups variables of RV1 and OPV shedding, only the variable with the lowest p value was included. Manual stepwise backwards elimination was used. At each step, the variable with the highest p value was eliminated until only variables with p values < 0.10 remained. Protective poliovirus-specific neutralizing antibodies at 12 weeks was not included as a variable as this was determined only for a small subset of participants.

Only RV1 shedding and OPV shedding after 1st dose RV1 remained significant predictors in multivariable analysis. The Bayesian Information Criterion (BIC) statistic was used to compare each iteration of the model and confirmed that the final model provided the best fit.

Table 4-9: Predictors of seroconversion: multivariable analysis

Predictor	Univariable RR (95%CI)	Multivariable Adjusted RR (95%CI)
RV1 shedding after 1 st dose RV1	3.1 (1.9-5.1) $p < 0.001$	2.2 (1.3-3.9) $p = 0.005$
OPV shedding in the 1st dose period	0.46 (0.26-0.82) $p = 0.009$	0.51 (0.32-0.82) $p = 0.005$
Non-secretor phenotype	0.49 (0.22-1.1) $p = 0.08$	0.35 (0.11-1.1) $p = 0.07$
Log RV specific IgG	0.81 (0.65-1.0), $p = 0.08$	NA

4.5 DISCUSSION

Seroconversion in Malawian infants following RV1 immunisation at 6 and 10 weeks

As expected, RV-specific IgA seroconversion in this population was low at 24% (18-31%). This is similar to seroconversion rates reported following the same vaccine schedule in other low-income settings such as Vellore, India, where seroconversion was 31% (Lazarus et al., 2017). RV-specific IgA GMC was also similar to GMCs reported in Vellore, South Africa and Ghana using a 2-dose 6/10 week schedule (Lazarus et al., 2017, Armah et al., 2016, Steele et al., 2010).

Baseline seropositivity (RV-specific IgA $> 20 \text{ U/l}$) at 6 weeks was 6%; similar to baseline seropositivity rates in the Malawi vaccine trial (10%), higher than in Ghana ($< 1\%$) but much

lower than in Vellore (30%)(Madhi et al., 2010, Armah et al., 2016, Lazarus et al., 2017).

Variation in seropositivity at 6 weeks reflects neonatal exposure to rotavirus and highlights that not all low- and middle-income settings are directly comparable, with a much higher burden of rotavirus exposure in some settings.

Post-immunisation seropositivity (RV-specific IgA >20U/mL) in this cohort was only 24% (19-31%), similar to results following a comparable vaccine schedule in Ghana (seropositivity 29% (22-37%)) (Armah et al., 2016). Of note, seropositivity in this cohort was much lower than in the Malawi vaccine trial (seropositivity 47% (30-66%)(Madhi et al., 2010). Direct comparison between the current and Malawi trial cohort is difficult due to a number of differences. Firstly, the two-dose vaccine trial schedule was given later at 10 and 14 weeks, which may be associated with increased immunogenicity (Armah et al., 2016, Steele et al., 2010). This later schedule also determined that post-immunisation samples were taken later (at a mean age of 16 weeks) compared to 12 weeks in the current cohort. Secondly, in the trial, only a minority of participants had baseline bloods, therefore infants seropositive at baseline were not excluded. This could increase the post-immunisation seropositivity rate by up to 10%. Finally, seropositivity in the placebo group was extremely high (40%). This suggests high background exposure to natural rotavirus infection, which may have contributed to seropositivity in the vaccine arm. In the current cohort, lower overall seropositivity rates suggest that in the context of high vaccine coverage, exposure to rotavirus in early life appears to be much lower. This likely reflects indirect effects of universal rotavirus vaccination in Malawi (Bennett et al., 2016, Bar-Zeev et al., 2016). Comparably low seropositivity rates in this study to studies in Ghana and South Africa provides supportive evidence to the conclusions of Armah et al. (2016) and Steele et al. (2010), that immunogenicity of the WHO recommended 6 and 10 week two dose schedule is low. Optimisation of vaccine scheduling by later administration or addition of a booster dose could potentially improve immunogenicity and vaccine effectiveness (Cunliffe and Kang, 2016).

The relationship between RV1 shedding and RV1 seroconversion

RV1 shedding, particularly following 1st dose was strongly associated with seroconversion, both by categorical and quantitative comparisons. The association was less clear for RV1 shedding

following the 2nd dose, and infants who shed only after the 2nd dose were less likely to seroconvert. This may be due to the time point of IgA sampling 6 weeks following 1st RV1 dose, but only 2 weeks following 2nd dose. This timepoint was chosen based on the peak rise of IgA in challenge studies (Bernstein et al., 1989), but is earlier than in some other studies. Sampling too early could potentially miss seroconversion in infants following the 2nd dose. The RoVi study, a cohort study expanding on this work in Malawi, has a post-immunisation blood sampling time point 4 weeks following the 2nd dose and should answer whether earlier sampling makes a significant difference to seroconversion results (Sindhu et al., 2017). Although this potential limitation must be considered, there are also immunological mechanisms which could account for this variation in the association between shedding and seroconversion for each dose.

In children naïve to rotavirus, shedding after 1st exposure to RV1 likely represents effective vaccine virus replication and therefore predicts subsequent seroconversion. Potential reasons for failure to shed RV1 following the 1st dose, such as inhibition by maternal antibody, are discussed in Chapter 3. Some of these inhibitory effects, such as maternal antibody, may have diminished by the 2nd dose at 10 weeks. In this case, RV1 shedding following the 2nd dose could also reflect effective vaccine take, and would predict seroconversion. However, in some infants persistent shedding at this stage may reflect failure to control vaccine virus replication, due to an ineffective mucosal immune response to the 1st dose. This would predict failure to seroconvert. The overall effect would be to move the association between RV1 shedding following the 2nd dose and seroconversion toward the null, as was seen in this study. The complexities of the relationship between shedding and immune response are discussed further in relation to OPV shedding below.

The clear confirmation of an association between RV1 vaccine virus shedding overall and seroconversion is of both conceptual and practical importance. Correlation, both as a quantitative and categorical variable, with RV-specific IgA response, the most widely accepted surrogate of protection, supports the concept that vaccine virus faecal shedding is a valid measure of vaccine take. From a practical perspective, vaccine shedding as an alternative measure of vaccine take to seroconversion has significant advantages. Stool sampling is non-invasive and therefore more ethically and socially acceptable. Molecular methods to detect

shedding are faster, more reproducible, and more suitable for high-throughput batch testing than ELISA methods required for serology. Immunogenicity studies tend to be restricted by the ethical and logistical limitations to smaller studies or sub-groups, shedding studies could potentially be undertaken at much larger scale. The current shedding data was derived from 4 sampling time points per dose, which increases sensitivity but increases cost and logistical challenges. However, as discussed in Chapter 3, sample collection at key time points (in this cohort, day 6) was reasonably predictive of shedding overall. In support of this principle Lazarus et al (2017) used shedding of >100 copies at day 4 or 7 following the 1st dose of RV1 only as an outcome measure in a trial of the effects of probiotics and zinc on vaccine response. They reported RV1 shedding rates following 1st dose of 24%, only slightly lower than my study, and also identified a strong correlation with seroconversion. Further studies in different populations to determine optimal sampling and potentially identify appropriate viral load cut-offs for shedding could increase the accuracy of this measure.

The relationship between passively-acquired maternal antibody and RV1 seroconversion

There was some evidence to support the hypothesis that higher passively-acquired maternal antibody (RV-specific IgG) at time of immunisation would be associated with reduced RV-specific IgA response. RV-specific IgG at baseline was higher in those who did not seroconvert but not significantly so. There was a weak negative linear correlation between rotavirus-specific IgG and post-immunisation RV-specific IgA. Analysis by quartile showed lower post-immunisation RV-specific IgA and a borderline significant negative effect on seroconversion only for infants with the highest quartile of RV-specific IgG compared to those in the lowest quartile. This suggests a threshold effect, where only very high levels of RV-specific IgG will adversely affect RV-specific IgA response. Similar effects were noted by Armah et al. (2016) in Ghana, who reported a seroconversion rate of 39% in infants with IgG <25th centile, compared to 6% in infants with IgG >75th centile ($p=0.001$). Chilengi et al. (2016) in Zambia reported a similar, although non-significant, trend.

Siegrist (2003) convincingly argues that if the primary mechanisms of maternal antibody inhibition of infant vaccine response are epitope masking by IgG preventing B cell priming, or

increased uptake of maternal antibody-antigen complexes by antigen presenting cells, then the maternal IgG/antigen ratio is the main determinant of infant vaccine response. Inhibition would only occur above a certain threshold level of maternal antibody, and as T cell priming is unaffected, would allow a normal vaccine response with subsequent dosing. Although this mechanism was primarily proposed to explain maternal antibody inhibition of parenteral vaccine response, epitope masking could similarly occur in the submucosa and inhibit intestinal B cell responses. There is evidence to support a threshold effect in inhibition by maternal antibody from studies of parenteral vaccines (Dagan et al., 2000, Jones et al., 2014). Passively-acquired IgG shows a consistent linear decline over the first 6 months of life. Dagan et al. (2000) in a study of Hepatitis A vaccination in Israel, showed a predictable vaccine response at each of 3 vaccine doses when infants were stratified by baseline IgG. Infants with higher initial IgG failed to respond to initial doses, but all infants adequately responded once maternal antibody had fallen below a threshold of 300-400 U/mL. A similar threshold effect may be seen for oral vaccines. Appaiahgari et al. (2014) in an immunogenicity dose-finding study of the oral rotavirus vaccine ORV-116E found evidence for a threshold effect, with the lowest levels of maternal IgG associated with the highest levels of infant IgA response. For RV1 rotavirus vaccine, this threshold effect may explain the findings of Moon et al. (2016) in South Africa, who reported an inhibitory effect of RV-specific IgG at baseline on seroconversion following 1st dose of RV1 at 6 weeks, but no effect on of baseline IgG on seroconversion following the 2nd dose at 14 weeks, by which time passively-acquired IgG would have significantly declined. In Chapter 3, RV1 vaccine virus shedding was found to be lower in infants with the highest quartile of RV-specific IgG, but only following the 1st dose of immunisation. It may be that by the second dose of immunisation, RV-specific IgG had declined below the inhibitory threshold, allowing an adequate RV-specific IgA response to second dose. This could explain the weaker negative association observed between RV-specific IgG and seroconversion, compared to the relationship with RV1 vaccine virus shedding.

A threshold effect could also partially explain observed differences in immunogenicity to different rotavirus schedules in different settings. For example, in settings with relatively low rotavirus burden/low maternal antibody a two-dose schedule, even if the initial dose is given at

6 weeks, would be predicted to be highly effective, since the majority of infants will have passively-acquired IgG below the inhibitory threshold, and a three-dose schedule would show no additional benefit. For infants in a medium burden/medium maternal antibody setting, overall immunogenicity would be predicted to be more modest, but a three-dose schedule or delayed dosing would have benefit, as infants above the inhibitory threshold for the 1st dose may fall below it by the 2nd or 3rd. An example of this might be in the delayed second dose in the South African study discussed above (Moon et al., 2016), or in Ghana, where baseline RV-IgA seropositivity of <1% in infants suggested a moderate burden of rotavirus, and where a 3 dose schedule at 6/10/14 weeks proved more immunogenic than a 2 dose schedule at 6 and 10 weeks (Armah et al., 2016). In countries with a higher burden/higher maternal antibody setting, a majority of infants might still remain above the inhibitory threshold at 14 weeks, predicting low immunogenicity and no benefit of an additional dose at this point. An example might be Pakistan, with baseline infant RV-IgA seropositivity of 8%, where a 3-dose regime was of no benefit (Ali et al., 2014). Similarly, in very high burden settings passively-acquired IgG might remain above the inhibitory threshold until well into the first year of life. This might partly explain why in one study in South India, where baseline infant RV-IgA seropositivity of 58% suggested very high burden, 54% of infants failed to seroconvert even after a 5th dose was given at 22 weeks (Kompithra et al., 2014). This concept needs tested further with studies analyzing the response to different dosing schedules stratified by baseline passively-acquired RV-specific IgG response. However, this supports the view that a “one schedule fits all” approach is unlikely to be successful in improving rotavirus vaccine efficacy in low- and middle-income countries.

The hypothesis that maternal antibody/antigen ratio is a key determinant of the IgG-mediated inhibition of vaccine response would also predict that increasing antigen dose/viral load could potentially overcome the effect. There is evidence for this for hepatitis A and measles vaccines, where increasing antigen dose/viral titres has been shown to overcome the effect of maternal inhibition (Dagan et al., 2000, Cutts et al., 1994). This has also been shown for the new Indian rotavirus vaccine ORV-116E, where an inhibitory effect of RV IgG on lower titre vaccine formulations was overcome by increasing the vaccine titre to 10⁵ FFU (Appaiahgari et al., 2014). However, increasing titre in live vaccines is not without risk, as in the case of measles vaccine

where high titres were associated with non-specific mortality effects (Knudsen et al., 1996). Careful safety and immunogenicity studies would be required to assess the risk:benefit of this strategy.

Relationship between OPV vaccine response and RV1 seroconversion

We found limited evidence for the hypothesis that robust OPV vaccine response would be associated with reduced RV1 seroconversion. OPV shedding in the 1st dose period was associated with reduced RV1 seroconversion, but OPV shedding in the 2nd dose period was not. The observed associations did not differ when OPV shedding was stratified by Sabin sub-type. We found no association between attainment of polio-specific neutralizing antibody response to serotypes 1 and rotavirus vaccine seroconversion. There was a positive association between attainment of polio-specific neutralizing antibody response to serotype 3 and rotavirus vaccine seroconversion – all infants who failed to attain poliovirus serotype 3 protective titres also failed to seroconvert to rotavirus vaccine. This is in contrast to data from Parker et al. (2018) in Vellore, who found no significant association between type 3 poliovirus neutralizing antibody and RV1 seroconversion.

As described in detail in Chapter 1.3.4.5, several previous studies have reported evidence of an inhibitory effect of concurrent OPV immunisation and rotavirus vaccine antibody response. However, some of these studies were limited by small sample size, with non-statistically significant trends rather than strong evidence for an inhibitory effect (Steele et al., 2010, Zaman et al., 2009). Others were potentially biased by post-hoc or opportunistic study designs (Emperador et al., 2016, Patel et al., 2012, Ramani et al., 2016). Several studies found some evidence that concurrent OPV was associated with reduced rotavirus immunogenicity, but that this effect did not reduce overall rotavirus seroconversion rates following completion of a 2 or 3 dose schedule (Rennels et al., 1996, Ciarlet et al., 2008, Steele et al., 2010). Only one study assessed the impact on vaccine efficacy. Tregnaghi et al. (2011) compared rotavirus vaccine efficacy in infants in Latin America who received concurrent OPV to infants in a historical control group in a similar population who did not receive OPV and found no difference in

clinical protection. Current evidence of a direct inhibitory effect of OPV on rotavirus vaccine virus response is far from conclusive.

In Chapter 3, we found no evidence for a direct inhibitory effect of OPV shedding on RV1 shedding. It was proposed that the complex relationships observed between OPV and rotavirus shedding and immunogenicity could instead be explained within a conceptual framework by common factors determining patterns of response to both vaccines, rather than by direct competitive inhibition (Figure 4-5). Alternatively, the mechanism of inhibition of concurrent OPV could be immune mediated. This would be supported by the finding that despite evidence for direct competition between OPV and RV1 vaccine virus shedding, OPV vaccine response as measured by OPV shedding in the first dose period, and to a lesser extent the second dose period, was associated with a reduction in rotavirus vaccine seroconversion. OPV could be immunodominant over rotavirus vaccine, possibly due to the advantage of T-cell priming effects from the birth dose of OPV.

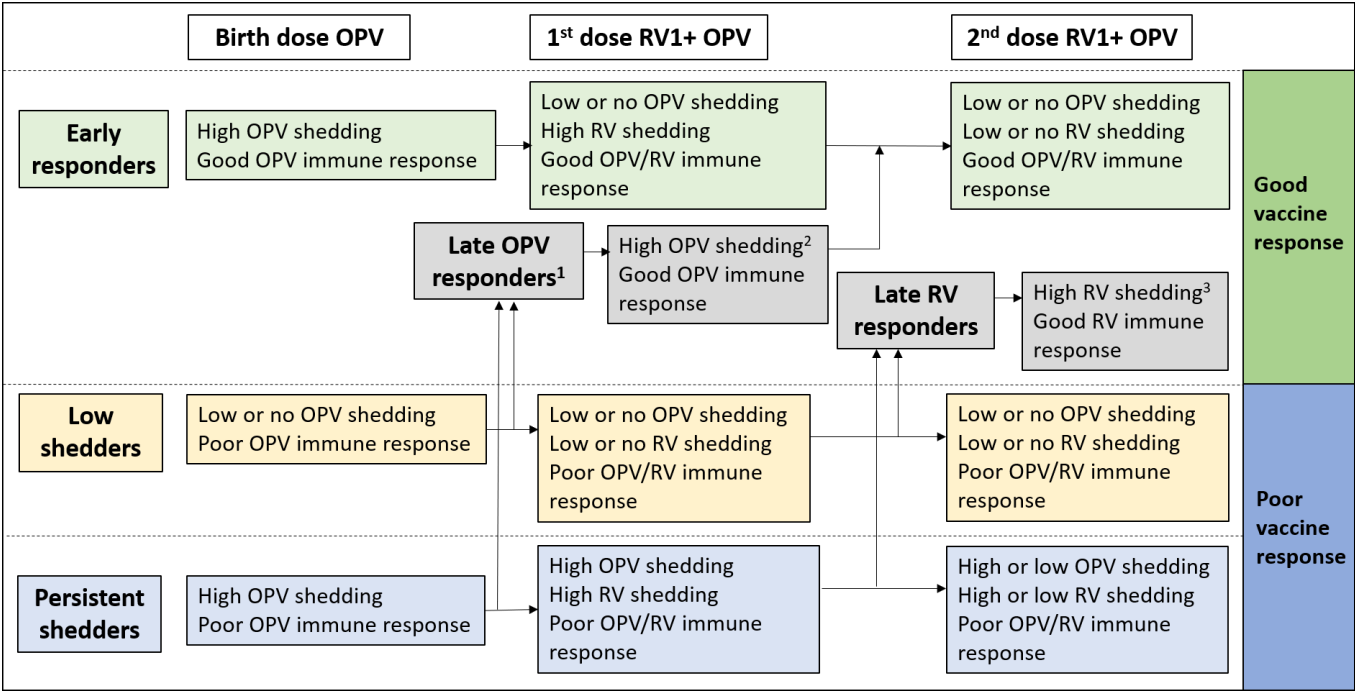


Figure 4-5: Conceptual framework of OPV and rotavirus vaccine response
 1. Late OPV response may also be delayed until dose 3 or later 2. RV shedding in late OPV responders may be variable, dependent on any inhibitory factor and its effect on RV1 response 3. OPV shedding in late RV responders may similarly be variable

As discussed in Chapter 3, the positive association between protective titres to type 3 poliovirus at 6 weeks and RV1 shedding in the first dose period could be explained by a sub-group of early responders, with rapid and optimal response to both live oral vaccines. This concept that infants could respond similarly to both oral vaccines is supported by the positive association observed between protective titres to poliovirus serotype 3 and rotavirus vaccine seroconversion. All 14 infants who failed to attain protective NA titres to poliovirus serotype 3 also failed to achieve rotavirus vaccine seroconversion. A similar relationship was not noted for serotype 1 poliovirus responses, but the Sabin 3 vaccine virus is known to be the least immunogenic within the trivalent vaccine. It may be that common factors predict poor response to both Sabin 3 and rotavirus vaccines.

As discussed in Chapter 3, my OPV data is limited by an observational study design and small sample size. The restrictions following the global switch to bivalent OPV limited my opportunity to assess Sabin 2 serology. Results and conclusions should be considered speculative. However, I believe this data presents an interesting challenge to current assumptions about both the mechanism and extent of any inhibitory association between OPV and rotavirus vaccine responses. I plan to combine my study data with OPV data collected as part of the ongoing RoVi study in Malawi and Vellore. This should enable further confirmation of these key findings and explanatory concepts.

Relationship between HBGA phenotype and RV1 seroconversion

Despite the evidence for a possible positive association between secretor phenotype and RV1 vaccine virus shedding described in Chapter 3, we found no association between seroconversion or any other measure of RV-specific IgA response and Lewis or secretor phenotype. This is in keeping with Bucardo et al. (2018), who also found no significant relationship, but in contradiction to Kazi et al. (2017) in Pakistan who reported lower seropositivity following 3 doses of RV1 in non-secretors: 10/53(19%) non-secretor infants seroconverted, compared to 46/127(36%) secretors (Fisher's exact $p=0.02$). Study sample size in the cohort was slightly larger than Kazi et al. (2017), but seropositivity rates were lower. The cohort study was adequately powered to detect a risk ratio of 0.5/2.0 between secretor and

Lewis HBGA phenotypes for the primary outcome measure of seroconversion, but may have failed to detect smaller effect sizes, particularly in relation to some of the less common HBGA phenotypes.

Bucardo et al. (2018) included data from 10 Le^{a+b-} Nicaraguan infants, none of whom seroconverted following RV1 immunisation. The authors concluded that Lewis A phenotype inhibits RV1 vaccine response. My study population included over twice as many infants of this phenotype, 4/21(19%) of whom seroconverted. Further studies with larger numbers are required to resolve these discordant results.

Both Kazi et al. (2017) and Bucardo et al. (2018) reported an effect of ABO phenotype on vaccine response, although they reported different associations. Kazi et al.(2017) reported increased seroconversion in blood group O (20/39, 51%), compared to non-O (26/88, 30%), but no significant difference when type O was compared to each of the non-O blood types separately. No such association was observed in this Malawian population where 27/86(31%) of non-O infants seroconverted compared to 14/65(22%) type O infants. Bucardo et al. (2018) reported lower seroconversion, 1/19 (5%), in type B infants, than in other ABO types. In the Malawian cohort, 9/30 (30%) type B infants seroconverted, with no difference in seroconversion compared to other ABO phenotypes. All three studies, including this study, are arguably underpowered for sub-group analysis by ABO phenotype. All of these results should therefore be interpreted with caution.

The evidence for an effect of HBGA phenotype on RV1 vaccine virus shedding was relatively weak. It is possible that RV1 vaccine virus shedding is relatively lower in non-secretor infants, but still sufficient to generate an adequate immune response.

It is clear, from this study and from Kazi, Cortese et al. (2017) and Bucardo et al. (2018), that any inhibitory effect of HBGA phenotype on RV1 vaccine response is not absolute. Absolute inhibition of vaccine response determined by HBGA phenotype was always unlikely, particularly for the non-secretor phenotype. Unlike the Lewis phenotype, the prevalence of non-secretor phenotype is consistent at around 20% across African and European populations. If all non-

secretor infants were intrinsically resistant to RV1, observed seroconversion rates in the high 90's in European countries would have been highly improbable (Patel et al., 2013).

In the wider context of the consistent evidence that non-secretor and Lewis negative infants are protected against G1P[8] rotavirus gastroenteritis, it is more likely that these phenotypes confer partial resistance to G1P[8] virus replication, and by extension RV1 vaccine virus replication. Although *in vitro* saliva binding and infectivity studies suggested specific HBGAs were the primary receptors for genotype-specific rotavirus binding, it is possible that *in vivo* there are secondary receptors which are less efficient but still adequate for virus attachment and replication. Alternatively, as suggested by Bohm et al. (2015) who challenged the results of previous *in vitro* studies, the relationship with HBGA phenotype and susceptibility may be indirect. Binding *in vivo* may occur to other similar carbohydrate antigens, which may also be determined by the FUT2 gene.

Partial resistance to P[8] infection would explain a modest reduction in RV1 vaccine virus replication, but allow adequate vaccine immune response. This new hypothesis would predict that non-secretor or Lewis negative infants would be protected against P[8] gastroenteritis, since lower viral loads are associated with less severe disease, but remain susceptible to asymptomatic P[8] infection. This hypothesis will be explored further in Chapter 5: Clinical Protection.

Relationship between seroconversion and other factors.

We identified no association between RV1 seroconversion and low birth weight or any measure of nutritional status at time of immunisation. This is consistent with data from the PROVIDE study in Bangladesh, a large prospective cohort study examining the effect of multiple factors on oral rotavirus responses and child growth and development (Naylor et al., 2015). RV1 was given at 10 and 17 weeks and RV-specific IgA response measured at 18 weeks. No association was found between nutritional status at baseline and RV-specific IgA or OPV response. Interestingly, better nutritional status at baseline was associated with a better response to all other EPI vaccines.

We identified no association between HIV exposure and RV-specific IgA conversion. My results are consistent with previous immunogenicity studies showing no evidence of sub-optimal RV1 vaccine response in HIV-infected children (Steele et al., 2011).

No association was found between any of the measured markers of poverty and poor sanitation and RV1 seroconversion. As discussed in Chapter 3, the distribution of poverty and poor sanitation in this population was similar to reported demographics for urban Malawi. As discussed in Chapter 3 lack of observed associations may reflect a “floor” effect in this very poor population. However, the results are consistent with the large and complex PROVIDE cohort study in Bangladesh (Naylor et al., 2015) which looked in detail for markers of poverty and lower socioeconomic status and found that only higher maternal education was significantly associated with rotavirus vaccine success.

Limitations

The potential for recruitment bias due to high attrition is discussed in detail in Chapter 3.

Exposure to wild type rotavirus contributing to rotavirus-specific IgA rise during the immunisation period cannot be excluded in the current cohort. This could lead to an over-estimation in vaccine-associated seroconversion. Establishing the incidence of wild-type rotavirus infection during the immunisation period is problematic, since there was no available simple molecular method to determine wild-type rotavirus which would not also be positive for vaccine virus. Genotyping was not possible in the majority of samples due to low viral load. However, given overall much lower seropositivity in the current cohort compared to the Malawi vaccine trial, it seems likely that exposure to wild-type infection in early infancy may be much lower than in the pre-vaccine era (Bar-Zeev et al., 2016). It should also be noted that although 30% of infants were shedding wild-type rotavirus at 6 weeks old, suggestive of recent exposure, only 6% of infants were seropositive. As suggested in Chapter 3, this could indicate that low viral load rotavirus infection in early infancy may not generate a robust rotavirus-specific IgA response. Alternatively, it could suggest that the IgA ELISA lacked sensitivity. High levels of IgG, which has higher avidity than IgA, could have competed for binding leading to falsely lower IgA concentrations. However, this was the same methodology used in the Malawi vaccine trial and

used extensively in other studies (Madhi et al., 2012, Paul et al., 2014). Very high levels of IgA were detected in some infants, and in a high proportion of infants in the case:control study (see Chapter 5) suggesting inhibition was not a major factor. Even if inhibition exists, the relative comparison of IgA response between participants in this study was of more interest than the absolute values.

The proportion of infants with rotavirus vaccine seroconversion was lower than that predicted when determining sample size. This could have reduced power to determine some outcomes, particularly in relation to HBGA phenotype and to OPV vaccine responses. OPV analysis was further limited by reduced sample size due to the unavoidable challenge of the global switch to bivalent OPV in April 2016.

Limitations of HBGA phenotyping are discussed in Chapter 3.

Summary

Both my data showing a potential threshold inhibitory effect of maternal antibody, and my conceptual framework of oral vaccine virus shedding and immune response, may have important implications for design of booster immunisation strategies. This will be discussed further in Chapter 6.

Although highly informative, both vaccine virus shedding and RV-specific IgA response are proxy markers for the most important measure of RV1 vaccine response: clinical protection. Predictors of clinical protection will be discussed in Chapter 5.

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5 PREDICTORS OF CLINICAL PROTECTION

5.1 INTRODUCTION

Efficacy of RV1 against severe rotavirus gastroenteritis in Malawi was only 49% (95%CI 19-68%), amongst the lowest efficacy reported for RV1 in trials worldwide (Madhi et al., 2010). Despite low efficacy, the high burden of rotavirus disease in Malawi predicted that vaccination would still have significant public health impact, and RV1 was introduced into the Malawi national immunization schedule in 2012. High vaccine coverage was rapidly achieved, with over 90% coverage maintained from 2014 onwards (Bar-Zeev et al., 2016). Post-introduction vaccine effectiveness studies have been more promising than trial outcomes, with estimated vaccine effectiveness in the first year of life of up to 70% (95%CI 34-87%) and a significant reduction in the incidence of rotavirus hospitalization in infants over time (Bar-Zeev et al., 2016).

Despite this success, vaccine effectiveness remains lower in Malawi than in higher income settings, and there is a persistent burden of severe disease, with associated mortality. All deaths from rotavirus should be considered preventable. Understanding factors contributing to reduced clinical protection from rotavirus vaccine therefore remains a priority.

Clinical vaccine failure may be primary, due to failure to generate an initial protective immune response, or secondary, due to waning immunity over time. The observed incidence of clinical vaccine failure will also be dependent on ongoing exposure, “force of infection”, within a population and clinical susceptibility to severe disease.

Potential contributory factors to primary vaccine failure, including the inhibitory effect of maternal antibody, concurrent OPV immunization, and the effect of enteric co-infection and poor gut health have been discussed in Chapters 3 and 4 (Figure 5-1). As discussed, these factors may be mediated through reduced vaccine virus replication (e.g. HBGA phenotype), reduced immunogenicity (e.g. concurrent OPV) or both (e.g. inhibition by maternal antibody).

The most common cause of secondary vaccine failure for any vaccine is a natural decline in immunity over time. The extent and rate of decline varies, may be dependent on boosting

through continued exposure to infection, and is reflected in the varying need for and timing of booster doses for different vaccines and in different populations (Riolo and Rohani, 2015).

The extent and rate of decline in protective immunity against rotavirus following immunization is unknown. The dynamics of RV-specific IgA following immunization have been little studied beyond the initial vaccination period. Lower vaccine efficacy/effectiveness in the second year of life may reflect secondary vaccine failure. In Malawi, there is evidence for reduced clinical protection in the 2nd year of life: vaccine efficacy against severe rotavirus gastroenteritis dropped to 18%(-59-56%) in the second year of life (Cunliffe et al., 2012). A similar decline was observed in vaccine effectiveness, which was reported as only 32%(-141-81%) in children in their second year (Bar-Zeev et al., 2016). Similar declines in clinical protection beyond one year of age have been reported in some middle income settings in Latin America and sub-Saharan Africa (de Palma et al., 2010, Justino et al., 2011, Correia et al., 2010, Armah et al., 2016), but not in others (Gastanaduy et al., 2016a, Pringle et al., 2016, Groome et al., 2014). Studies in higher income countries have been more consistent, with vaccine effectiveness sustained into the second year and beyond (Karafillakis et al., 2015, Phua et al., 2012). Debate continues regarding the extent to which the apparent decline in clinical protection reflects waning immunity, the interaction between waning immunity and force of infection, or the limitations of observational study designs (Lopman et al., 2012). It should also be noted that very few studies were adequately powered to examine age-related vaccine efficacy/effectiveness, reflected in imprecise reported estimates.

While accepting these limitations, it is biologically plausible that rotavirus vaccine-related protective immunity will decline over time, as it does for other vaccines such as tetanus, diphtheria and pertussis which for optimal protection require boosters in early childhood. In populations with sub-optimal vaccine response, infants have a lower post-vaccination RV-specific IgA (Patel et al., 2013). Assuming a consistent rate of decline in RV-specific IgA post-immunisation, levels in infants with a sub-optimal vaccine response might fall below the threshold of IgA associated with protection, sooner than infants with a higher baseline RV-specific IgA response. A more rapid decline in mucosal immune response might be observed in infants with acquired conditions causing systemic or mucosal immune dysfunction— such as

malnutrition, HIV/AIDS or environmental enteropathy (Figure 5-1). These conditions become more clinically prevalent in later infancy. However, the dynamics of RV-specific IgA post-immunisation are not well understood. There may not be a consistent decline, particularly in the context of ongoing exposure to rotavirus infection (Premkumar et al., 2014). In addition, while RV-specific IgA is the best available surrogate of protective immunity, a protective threshold has not been established. In this chapter, I will explore the extent to which RV-specific IgA predicts vaccine failure, by comparing RV-specific IgA in vaccinated infants presenting with severe rotavirus gastroenteritis, to age-matched vaccinated controls.

Incidence of clinical vaccine failure depends not only on immunity of an individual, but also exposure to infection. Between populations, higher force of infection may contribute to a higher burden of clinical vaccine failure for an equivalent population level of immunity. Within populations, risk factors for exposure to enteric infection such as overcrowding or poor sanitation, could contribute to a higher individual risk of clinical vaccine failure (Figure 5-1). In settings of high vaccine coverage and high efficacy, the introduction of rotavirus immunization may reduce force of infection within a population. This could result in herd protection for infants with a sub-optimal vaccine response, reducing the risk of clinical vaccine failure.

Although the impact of indirect vaccine effects in low-income settings with high force of infection and sub-optimal vaccine efficacy may be more limited (Bennett et al., 2016), this could potentially explain higher than expected vaccine effectiveness in Malawi compared to trial efficacy. However, Patel et al. (2010) caution that limitations in observational study designs may result in an over-estimation of total vaccine effectiveness where vaccine coverage varies within populations.

Risk of clinical vaccine failure also depends on an individual's susceptibility to severe disease, rather than mild disease or asymptomatic infection. Genetic factors may determine predisposition to rotavirus disease. As discussed in Chapters 1 and 3, HBGA phenotype has been associated with genotype-specific susceptibility to rotavirus gastroenteritis. In Chapter 3 and 4 we found some evidence that HBGA phenotype predicted vaccine virus replication, however vaccine virus shedding was still observed in non-secretor/Lewis negative infants, and seroconversion appeared unaffected. This suggests that resistance of non-secretor/Lewis

negative individuals to P[8] rotavirus infection is relative, rather than absolute. From this we might predict that these phenotypes may protect against P[8] rotavirus gastroenteritis, but not against asymptomatic infection. Since these infants remain susceptible to rotavirus gastroenteritis of other genotypes, the overall effect of non-secretor/Lewis negative phenotype on clinical vaccine failure will depend on the balance between protective effects against disease, and inhibitory effects on vaccine response (Figure 5-1). In this chapter, we will test these hypotheses by determining the effect of HBGA phenotype on genotype-specific risk of rotavirus infection, and overall risk of clinical vaccine failure.

In diarrhoeal disease, severity is not just determined by pre-existing immunity to specific infection, but also on gut epithelial integrity and overall health. A child with pre-existing feeding problems due to cerebral palsy, for example, is more likely to become severely dehydrated following rotavirus infection, than a more robust child. In Malawi, poor nutritional status is the most common determinant of poor child health, and severely malnourished children have little clinical reserve. However, the relationships between poor growth, malnutrition, gut integrity and risk of diarrhoeal disease are complex. In this chapter, I will explore the effect of nutritional status, HIV exposure, and proxy measures of poverty and poor sanitation on overall risk of clinical vaccine failure.

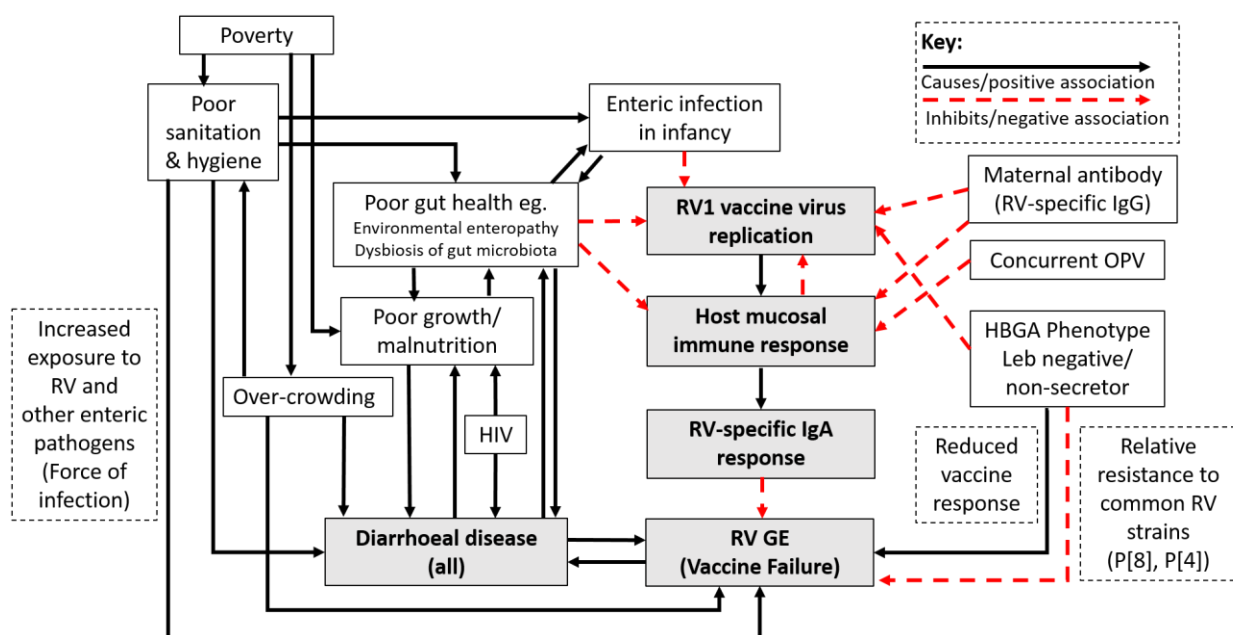


Figure 5-1: Proposed factors contributing to clinical vaccine failure

5.2 AIMS

To determine the effect of HBGA phenotype, RV-specific IgA concentration and socio-economic factors on odds of vaccine failure (defined as severe rotavirus gastroenteritis following two doses of RV1 vaccine).

5.2.1 Hypotheses

Primary Hypothesis

1. HBGA Phenotype

- a. Secretor status will determine strain-specific susceptibility to rotavirus gastroenteritis. Non-secretors will be less susceptible to P[8] and P[4] rotavirus strains.
- b. Lewis negative phenotype will determine strain-specific susceptibility to rotavirus gastroenteritis. Lewis negative infants will be less susceptible to P[8] and P[4] strains, but more susceptible to P[6] rotavirus strains.
- c. Non-secretor phenotype is associated with reduced RV1 vaccine take, and therefore increased risk of rotavirus vaccine failure with non P[8] rotavirus strains.
- d. Lewis negative phenotype is associated with reduced RV1 vaccine take, and therefore increased risk of rotavirus vaccine failure with non P[8] rotavirus strains.

Secondary Hypotheses

2. HBGA Phenotype

- a. Based on results from Chapter 3, showing reduced but not absent RV1 shedding in non-secretor infants, resistance to P[8] infection is relative, not absolute. Secretor/Lewis phenotype will not determine susceptibility to asymptomatic P[8] infection.

3. RV-specific IgA response

- a. Lower levels of RV-specific IgA will be associated with increased risk of rotavirus vaccine failure.

b. Infants with rotavirus vaccine failure will show a robust RV-specific IgA response to natural infection.

4. Infant host response

a. LBW infants will have increased risk of vaccine failure.

b. HIV exposed infants will have increased risk of vaccine failure.

c. Infants with poor nutritional status will have increased risk of vaccine failure

5. Socioeconomic and Sanitation factors

a. Predictors of poverty will be associated with increased risk of vaccine failure.

b. Predictors of poor sanitation will be associated with increased risk of vaccine failure.

5.3 METHODS

Recruitment and laboratory methods are summarized in detail in Chapter 2. Methods in brief were as follows:

5.3.1 Study design

This was a cross-sectional case: control study.

5.3.2 Study Population

Infants with severe gastroenteritis, defined as a Vesikari score ≥ 11 , were recruited from Queen Elizabeth Central Hospital; and from Zingwangwa, Gateway and Madizabango Health Centres, Blantyre. All included infants had received two doses of RV1 vaccine, confirmed by hand-held health records. Infants resident outside of Blantyre, or with known conditions causing immunosuppression (excluding HIV) were excluded. Stool from infants with severe gastroenteritis was tested for rotavirus by rapid immunochromatography test (ICT). Infants aged between 10 weeks and 1 year who were rotavirus positive were recruited as rotavirus gastroenteritis cases (vaccine failures). Infants who tested negative for rotavirus, were born within ± 30 days of rotavirus gastroenteritis cases, and had no known household contact with

rotavirus, were eligible as non-rotavirus gastroenteritis controls. Age-matched cases were matched 1:1 with non-rotavirus gastroenteritis controls.

Age-matched community controls, born within ± 30 days of rotavirus gastroenteritis cases, were recruited from randomly generated locations within healthcare catchment areas specific to each recruitment site. Community controls were also fully RV1 vaccinated, had no known condition causing immunosuppression (excluding HIV), had no history of diarrhoea within a week of recruitment, and had no known household contact with rotavirus. Age-matched cases were matched 1:1 with asymptomatic community controls.

A recruitment window of 90 days was allowed after recruitment of a rotavirus gastroenteritis case to identify age-matched non-rotavirus gastroenteritis and community controls, otherwise cases remained unmatched.

Sample size was based on the odds ratio of vaccine failure by HBGA phenotype and was calculated based on the estimated proportion of the phenotypes of interest (Lewis negative or non-secretors) in controls (Dupont, 1988). Assuming a minimum population prevalence of HBGA phenotype of interest of 20%, a sample size of 123 cases (plus 123 community and 123 non-rotavirus gastroenteritis controls) was estimated to achieve 80% power to detect an odds ratio of 2.5 versus the alternative of equal odds using a Chi-Square test with a 0.05 significance level.

5.3.3 Demographic, anthropometric and socioeconomic data collection

Social and demographic data were collected by structured interview. Confirmation of vaccination and timing of vaccine were taken from the child's handheld health record. Infants without a documented vaccine record were excluded.

Infant characteristics which could be associated with increased risk of clinical vaccine failure, including low birth weight and gender, were recorded as detailed in Chapter 2.6. A previous history of diarrhoea, resulting in either health centre attendance or hospital admission was included as a measure of overall diarrhoeal risk.

Infant and maternal HIV status was determined by verbal report from the carer, confirmed where possible from the child's hand-held health record. Where status was unknown, families were encouraged to attend for routine HIV testing and the result recorded. HIV-exposure was defined as any infant of an HIV-infected mother, or any infant under 12 months with a positive HIV antibody test. HIV-infection was defined as any infant over 12 months with a positive HIV antibody test, or any infant with a positive HIV DNA PCR test.

Nutritional status was determined by length, weight and mid-upper arm circumference (MUAC) measurement at time of recruitment, compared to WHO age-determined z scores. Dehydration results in transient weight loss. Severe dehydration is associated with 10% loss and some dehydration approximately 5%. This is quickly replaced with rehydration, and does not reflect underlying nutritional status. Most cases had some degree of dehydration and were weighed on admission, before rehydration. To avoid bias, weight of gastroenteritis cases was therefore adjusted by 5 or 10% to account for some or severe dehydration on admission respectively. MUAC was not adjusted for hydration status, since there is evidence that dehydration does not significantly alter MUAC in children with diarrhoea (Modi et al., 2015).

Key socio-economic data which could potentially be associated with higher risk of rotavirus transmission or diarrhoeal disease were included for analysis. Definitions of each are detailed in Chapter 2.6.

5.3.4 Laboratory methods

Laboratory methods are described in detail in Chapter 2.

Stool samples were taken from all cases and controls at recruitment, except for some infants from whom rectal swabs were obtained. Rotavirus gastroenteritis cases were confirmed by VP6 qRT-PCR. Non-rotavirus gastroenteritis controls and asymptomatic community controls were also tested for rotavirus by VP6 qRT-PCR. Rotavirus positive community controls were considered to have asymptomatic rotavirus infection. Non-rotavirus gastroenteritis (rapid ICT negative) controls were tested by rotavirus enzyme-immunoassay (EIA). EIA positive controls were excluded, and EIA negative controls retained. EIA negative non-rotavirus gastroenteritis controls were tested further by VP6 qRT-PCR: those with a VP6 cycle-threshold greater than 27

(the established cut-off for disease) were considered to have incidental rotavirus infection, with symptomatic gastroenteritis secondary to a different (unidentified) pathogen, and were retained as controls. Those with a VP6 cycle-threshold less than 27 were considered to have possible rotavirus gastroenteritis with false negative antigen tests and were excluded. All rotavirus cases and rotavirus infections with a cycle threshold of 35 or less were genotyped by hemi-nested PCR and gel electrophoresis.

HBGA phenotype was determined by salivary ELISA for A, B, H and Lewis a and b antigens. Infants who were negative for ABH were confirmed as non-secretors by Lectin salivary ELISA and by FUT2 genotyping. Infants who were positive for either Lewis a or Lewis b antigen were classed as Lewis positive, and those negative for both Lewis antigens as Lewis negative. Final determination of secretor and Lewis phenotype was made on the basis of two ELISA tests (ABH or Lectin) confirming the same phenotype. Analysis was made by unstratified and stratified Lewis/secretor HBGA phenotype.

RV-specific IgA was determined by sandwich ELISA. Controls had a single plasma sample taken at time of recruitment. Rotavirus gastroenteritis cases had both an initial sample at time of recruitment, and a convalescent sample taken as close as possible to 10 days following onset of diarrhoea.

5.3.5 Statistical Analysis

Summary statistics were described as mean and 95% confidence interval for normally distributed continuous variables, median and inter-quartile range for non-normally distributed continuous variables and proportion and 95% confidence interval of proportion for categorical variables.

To determine the relationship between HBGA phenotype and odds of genotype-specific rotavirus infection, only infants who had P type confirmed on genotype, or who were confirmed rotavirus negative on VP6 RT-PCR were included for analysis. Rotavirus VP6-PCR positive infants (either cases or controls) who could not be genotyped due to low viral load were excluded from this part of the analysis. The odds of a genotype-specific rotavirus infection (eg P[8]) compared to no rotavirus or rotavirus infection of a different genotype (eg P[6]) by HBGA phenotype were

determined by logistic regression. This analysis was unmatched. Non-rotavirus gastroenteritis controls were not included in HBGA and genotyping analysis due to the possibility of bias from other enteric infections associated with HBGA phenotype, such as norovirus.

To determine the relationship between HBGA phenotype and odds of clinical rotavirus vaccine failure, all rotavirus cases (of any genotype, or VP6 confirmed but not genotyped) were compared by conditional logistic regression to age-matched community controls by HBGA phenotype.

The remaining case-control analysis was made by conditional logistic regression comparing rotavirus cases to age-matched 1:1 with community and non-rotavirus gastroenteritis controls. To increase power, where there was no difference in a given variable between community and non-rotavirus gastroenteritis controls, controls were combined and compared 1:1 or 1:2 with rotavirus gastroenteritis cases, dependent on whether both matched community and non-rotavirus gastroenteritis controls were available.

In a separate analysis, rotavirus and non-rotavirus gastroenteritis cases were combined and compared to age-matched community controls by conditional logistic regression, to determine predictors of all cause gastroenteritis.

Multivariable models were created using conditional logistic regression. All variables significant to $p < 0.10$ in univariable analysis were included in the multivariable model. The model was built using a manual stepwise backward elimination approach, whereby variables with the highest p value were removed sequentially until only variables with $p < 0.1$ remained. The Benjamini-Hochberg procedure was applied to adjust for multiple comparisons, with a 25% false discovery rate (Benjamini and Hochberg, 1995). Akaike Information Criterion (AIC) were used to compare model iterations for goodness of fit. Statistical analysis was performed in StataC version 13.1 (StataCorp, US).

5.4 RESULTS

5.4.1 Recruitment of cases and controls

Recruitment began in January 2015 and ended in February 2017. Numbers screened and recruited are summarised in Figure 5-2. RV1 vaccine effectiveness was higher than expected, leading to a reduction in rotavirus gastroenteritis cases locally. A high proportion of both rotavirus gastroenteritis and non-rotavirus gastroenteritis cases in health centre sites did not meet severity eligibility criteria, or were infants over 12 months old, and 38% of potentially eligible cases declined to participate, most commonly because parents did not wish their child to have blood samples taken. This led to fewer than planned rotavirus cases being recruited (120 rather than 123). Recruitment of age-matched non-rotavirus gastroenteritis controls within the ninety-day recruitment window also proved difficult, particularly in the dry season when gastroenteritis is less common. As a result, only 95/120 cases were matched to a non-rotavirus gastroenteritis control. However, non-rotavirus gastroenteritis controls were primarily a control group for secondary (socioeconomic) predictors, and sufficient community controls were recruited for 1:1 matching on the primary outcome measure.

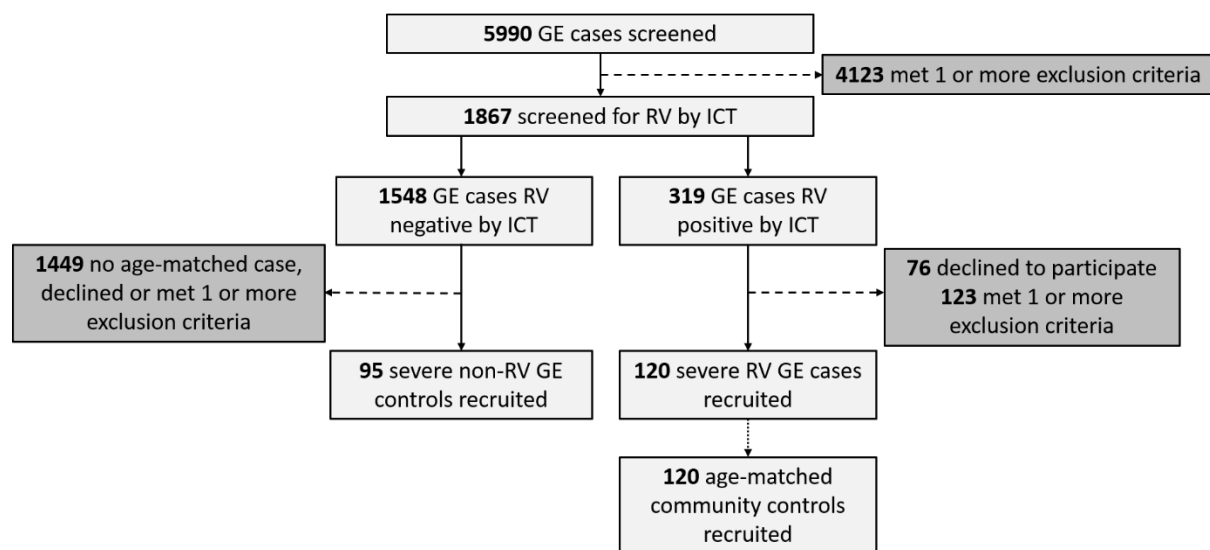


Figure 5-2: Screening and recruitment for case control study

Total numbers across all sites. ICT= rapid immunochromatography test. GE= gastroenteritis. RV GE= rotavirus gastroenteritis. Non-RV GE= rotavirus test negative gastroenteritis. Severe GE defined as Vesikari score ≥ 11 .

The median age of presentation with severe rotavirus gastroenteritis was 9.0 months (IQR 7.6-10.6 months) and with severe non-rotavirus gastroenteritis was 9.7 months (IQR 8.5-11 months). The median Vesikari score for severe rotavirus gastroenteritis was 14 (IQR 13-16) and for severe non-rotavirus gastroenteritis was 13 (IQR 12-15). Severe dehydration was more common in rotavirus gastroenteritis: 44/120, (37%, 28-46%) of severe rotavirus gastroenteritis cases were severely dehydrated compared to 14/95 (15%, 9-24%) severe non-rotavirus gastroenteritis cases. Three infants with severe rotavirus gastroenteritis and two infants with severe non-rotavirus gastroenteritis died.

5.4.2 Genotype of rotavirus gastroenteritis cases and asymptomatic RV infection

Three cases did not have stool available for confirmatory qRT-PCR, the remaining cases were all confirmed. Median rotavirus viral load was 1.4×10^7 (IQR 1.5×10^6 - 4.8×10^7) copies/mL.

Sixteen non-rotavirus gastroenteritis controls were ICT negative on rectal swab but did not have stool available for confirmatory EIA or qRT-PCR. The remaining non-rotavirus gastroenteritis controls were all confirmed negative by EIA, but 43/79 (54%, 43-65%) had low level rotavirus detected by VP6 qRT-PCR (median rotavirus viral load 170 (IQR 85-828) copies). As defined in the methods, these infants were considered to have incidental rotavirus infection below the cycle threshold associated with disease and were retained as controls.

Asymptomatic rotavirus infection (detectable rotavirus infection on qRT-PCR) was found in 77/120 (64%, 55-72%) of community controls. Viral load was significantly lower than in symptomatic cases, median rotavirus viral load in asymptomatic infection was 273 (IQR 77-1010) copies (Figure 5-3).

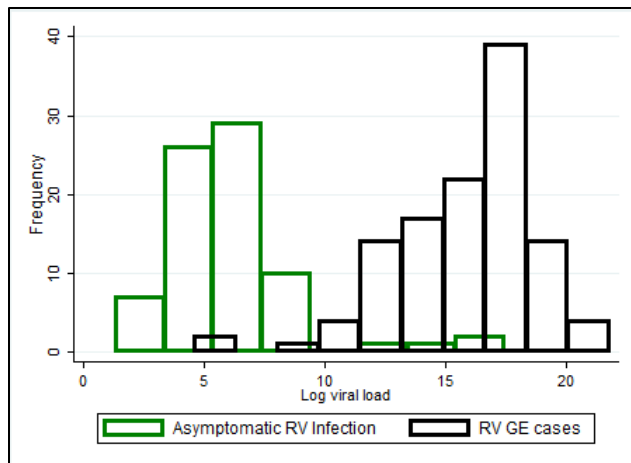


Figure 5-3: Rotavirus viral load in RV GE cases and asymptomatic RV infection

Histograms comparing distribution of viral load (VP6 copy number) in RV GE cases and asymptomatic RV infection (community controls). Viral load is log transformed for clearer visualization. Viral load in asymptomatic infection was significantly lower (Kruskal Wallis test $p < 0.0001$) than in RV GE.

Full or partial genotype was determined for 116/120 (97%) rotavirus gastroenteritis cases. The most common four genotypes accounted for over 75% of genotyped rotavirus gastroenteritis cases: G1P[8] (32%), G2P[4] (26%), G12P[6] (10%) and G2P[6](9%) (Figure 5-4:A).

Due to low viral load, only 21/77(27%) asymptomatic rotavirus infections could be fully genotyped, and 9/77(12%) partially typed. Three common genotypes accounted for over half of the genotyped asymptomatic infections: G1P[8] (23%), G2P[4](17%) and G12P[8](13%) (Figure 5-4:B). Less common genotypes and mixed infections in rotavirus gastroenteritis cases and asymptomatic rotavirus infection are summarized in Table 5-1. In addition, there were 7 partially typed P[4] infections, 3 partially-typed P[8] infections, 1 partially-typed P[6] infection, 2 partially typed G12 infections and 1 partially typed G2 infection.

In 115 gastroenteritis cases where P Type was confirmed, P[8] was identified in 48/115 (42%), P[4] in 34/115 (33%) and P[6] in 34/115 (29%) of infections, either alone or in mixed infection.

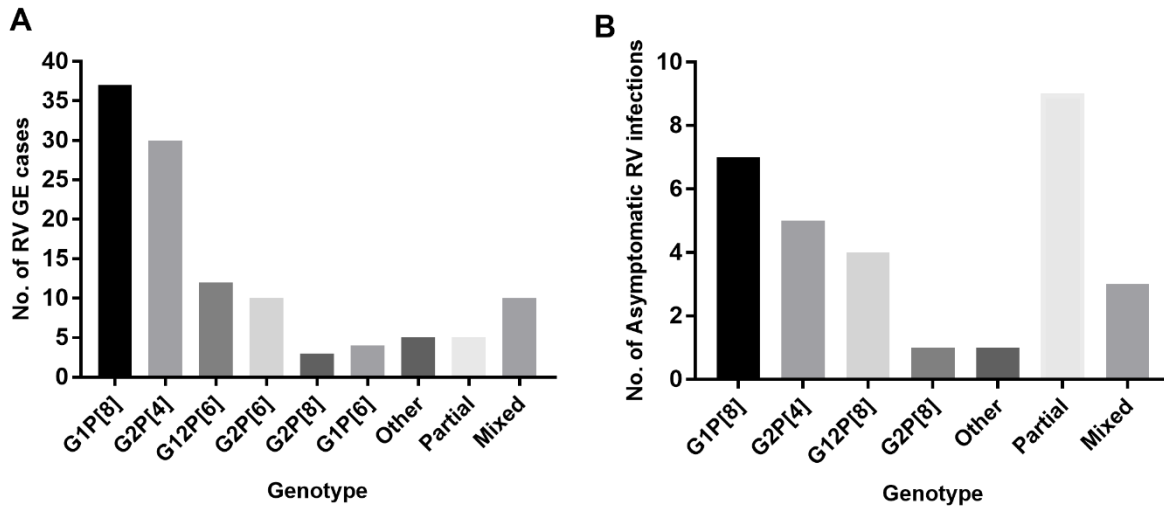


Figure 5-4: Common Genotypes in RV GE cases and asymptomatic RV infection
A: Common genotypes of RV GE cases B: Common genotypes in asymptomatic RV infection

Table 5-1: Uncommon rotavirus genotypes and mixed infections

Genotype	RV GE cases	Asymptomatic RV infections
Uncommon genotypes		
G1 P[4]	2	
G12 P[4]	2	
G12 P[14]	1	
G12P[10]		1
Mixed Infections		
G1 P[6]/P[8]	4	
G1/G2 P[14]	1	
G1/G2 P[8]	1	
G1 P[4]/P[6]/P[8]	1	
G1/G2/G12 P[6]	1	
G1/G2/G12 P[8]		1
G2/G12 P[6]	1	
G2 P[6]/P[8]	1	
G2 P[4]/P[8]		1
G9/G12 P[4]		1

5.4.3 HBGA phenotype and genotype-specific susceptibility to rotavirus GE

To determine the relationship between HBGA phenotype and genotype-specific susceptibility to rotavirus GE, the odds of a genotype-specific rotavirus infection (eg P[8]) compared to no

rotavirus infection or rotavirus infection of a different genotype (eg P[6]) by HBGA phenotype were determined. HBGA secretor and Lewis phenotypes were determined for 186 infants included for genotyping analysis: 114 P-typed rotavirus gastroenteritis cases, 29 P-typed asymptomatic rotavirus infections, and 43 rotavirus-negative asymptomatic community controls. Cases and asymptomatic community controls who were positive on rotavirus qRT-PCR but could not be genotyped were not included. The distribution of HBGA phenotype for this study sub-population is shown in Table 5-2.

Table 5-2: Distribution of secretor/Lewis phenotype in analysis of RV genotype and HBGA

	Lewis positive	Lewis negative	Total
Non-secretor	22	16	38 (20%)
Secretor	125	23	148 (80%)
Total	147 (79%)	39(21%)	186

The proportion of infants with saliva positive for both Le^a and Le^b was high: 69/117 (37%, 31-45%). Most Le^{a+b+} infants were phenotypic secretors: 63/148 (43%, 35-51%) secretors and 6/38 (16%, 7-32%) non-secretors were Le^{a+b+}. Three Le^{a+b+} phenotypically non-secretor infants were confirmed as non-secretors by FUT2 genotyping, one had a homozygous secretor genotype and two did not have blood available for genotyping.

Unexpectedly, 9/37 (24%, 13-41%) phenotypic non-secretors were Le^{a-b+}. Five of these infants were confirmed as non-secretors by FUT2 genotyping, two had secretor genotype (one homozygous, one heterozygous), and two had no blood available for genotyping.

The relationship between HBGA phenotype and odds of genotype-specific rotavirus infection was determined by logistic regression (Table 5-3).

Secretor phenotype increased the odds of P[8] gastroenteritis almost eight fold (Table 5-3). There was similar trend in the relationship between secretor phenotype and all P[8] infections, including asymptomatic infections, but this association was not significant. Asymptomatic infections, when assessed separately, were not significantly associated with secretor phenotype: 6/17(35%) asymptomatic P[8] infections (including 3/8 (38%) asymptomatic G1P[8] infections) were in non-secretors (Table 5-3).

Similarly, Lewis negative phenotype was associated with a 72% reduction in the odds of P[8] gastroenteritis, but a similar trend in the odds of any P[8] infection (including asymptomatic infection) was not significant (Table 5-3).

The relationship between secretor and Lewis phenotype and P[8] gastroenteritis was largely driven by the very strong association between secretor/Lewis phenotype and G1P[8] gastroenteritis. In a sub-analysis of 175 infants (including only infections where both G and P type were known and rotavirus negative infants) there were 52 G1P8 infections, including 44 cases of G1P[8] gastroenteritis. All 44 G1P[8] gastroenteritis cases were in secretors. A less strongly positive association was observed between secretor phenotype and odds of any G1P[8] infection: OR 5.7(95%CI 1.7-19.7), $p=0.005$. Lewis negative phenotype was associated with an 87% reduction in odds of G1P[8] gastroenteritis (OR 0.13, 95%CI 0.03-0.57, $p=0.007$), and 71% reduction in odds of G1P[8] infection overall (OR 0.29, 95%CI 0.11-0.79, $p=0.02$).

Table 5-3: Odds of genotype-specific RV infection by HBGA phenotype

	Secretor n=148 OR (95% CI)	Lewis negative n=39 OR (95% CI)
P[8] RV infection n=64	n=56 2.3 (0.98-5.3) $p=0.06$	n=9 0.50 (0.22-1.1) $p=0.10$
P[8] RV asymptomatic n=17	n=11 0.43 (0.15-1.2) $p=0.12$	n=5 1.7 (0.55-5.0) $p=0.37$
P[8] RV GE n=47	n=45 7.8 (1.8-33.7) $p=0.006$	n=4 0.28 (0.09-0.83) $p=0.02$
P[4] infection n=49	n=44 2.8 (1.0-7.6) $p=0.05$	n=4 0.26 (0.09-0.77) $p=0.02$
P[4] GE n=38	n=36 5.8 (1.3-25.2) $p=0.02$	n=2 0.17 (0.04-0.73) $p=0.02$
P[6] RV infection n=34	n=27 0.99 (0.39-2.5) $p=0.98$	n=13 3.0 (1.3-6.7) $p=0.008$

OR (95%CI)=odds ratio, 95% confidence interval. OR and p value determined by logistic regression.

**44/44 G1P[8] gastroenteritis cases were secretors. OR incalculable by logistic regression.*

Similarly, P[4] rotavirus gastroenteritis was also associated with secretor phenotype (Table 5-3). Odds of P[4] rotavirus gastroenteritis in secretor infants were increased almost six-fold. The association with all P[4] infection was less strong. Lewis negative phenotype was associated with an 83% reduction in the odds of P[4] gastroenteritis.

Lewis negative phenotype was associated with increased odds of P[6] rotavirus: Lewis negative infants were at three times increased odds of P[6] infection (Table 5-3). Almost all P[6] infections (33/34) were symptomatic, therefore could not be stratified by gastroenteritis and all infection.

Since secretor and Lewis phenotype are determined by the combination of FUT2 and FUT3 gene expression there is significant overlap between these phenotypes. To determine whether the combination of secretor and Lewis phenotype modified the observed associations, secretor and non-secretor infants were stratified by Lewis phenotype. Stratification by Lewis phenotype did not improve the predictive odds for P[8] infections, suggesting the association is largely driven by secretor phenotype (Table 5-4).

Table 5-4: P[8] rotavirus by secretor/Lewis negative phenotype

HBGA phenotype N=186	All P[8] RV infections n=64 OR (95%CI)		P[8] RV gastroenteritis n=47 OR (95%CI)	
<u>Non-secretor</u> n=38	n=7		n=2	
	0.44 (0.19-1.0)	p=0.06	0.13 (0.03-0.57)	p=0.007
<i>Lewis negative</i> n=16	n=4		n=1	
	1.5 (0.31-7.1)	p=0.61	1.4 (0.08-24.2)	p=0.82
<i>Lewis positive</i> n=22	n=3		n=1	
	0.67 (0.14-3.2)	p=0.61	0.71 (0.04-12.3)	p=0.82
<u>Secretor</u> n=148	n=56		n=45	
	2.3 (0.98-5.3)	p=0.06	7.6 (1.8-32.5)	p=0.007
<i>Lewis negative</i> n=23	n=5		n=3	
	0.40 (0.14-1.2)	p=0.09	0.30 (0.08-1.1)	p=0.06
<i>Lewis positive</i> n=125	n=51		n=42	
	2.5 (0.87-4.9)	p=0.09	3.3 (0.93-11.9)	p=0.06

OR (95%CI)=odds ratio, 95% confidence interval. OR and p value determined by logistic regression.

In infants with P4 infection, while overall secretor infants were at increased risk of infection, Lewis negative secretor infants were relatively protected compared to Lewis positive secretor infants (Table 5-5). A similar but inverse relationship was observed in stratified analysis of infants with P[6] infection, where Lewis negative secretor infants were at increased risk of P[6] infection compared to Lewis positive secretor infants (Table 5-5). However, for both P[4] and P[6] infections stratification did not improve predictive odds, compared to the predictive power

of Lewis negative phenotype alone. This suggests that these relationships may primarily be driven by Lewis, rather than secretor phenotype.

Table 5-5: P[4] and P[6] rotavirus by secretor/Lewis negative phenotype

HBGA Phenotype n=186	P[4] infection n=49 OR (95%CI)	P[6] infection n=34 OR (95%CI)
Non-secretor n=38	n=5 0.36 (0.13-0.98) p=0.05	n=7 1.0 (0.40-2.5) p=0.98
<i>Lewis negative</i> n=16	n=2 0.90 (0.13-6.2) p=0.92	n=3 1.0 (0.20-5.5) p=0.96
<i>Lewis positive</i> n=22	n=3 1.1 (0.16-7.5) p=0.92	n=4 0.96 (0.18-5.0) p=0.96
Secretor n=148	n=44 2.8 (1.0-7.6) p=0.05	n=27 0.99 (0.39-2.5) p=0.98
<i>Lewis negative</i> n=23	n=2 0.19 (0.04-0.84) p=0.03	n=10 4.9 (1.9-12.9) p=0.001
<i>Lewis positive</i> n=125	n=42 5.3 (1.2-23.7) p=0.03	n=17 0.20 (0.08-0.54) p=0.001

OR (95%CI)=odds ratio, 95% confidence interval. OR and p value determined by logistic regression.

FUT2 genotype was determined for 28/38 non-secretor infants. The remaining 10 infants did not have sufficient whole blood available. 7/28(25%) of phenotypic non-secretor infants tested (3 rotavirus gastroenteritis cases, 4 community controls) had secretor genotype (homozygous or heterozygous for the most common FUT2 polymorphism). One of these infants was Le^{a-b+} and one was Le^{a+b+}. From the community controls, these included 1 infant with P[8], 2 infants with P[4] and 1 infant with P[6] asymptomatic infection. From the rotavirus gastroenteritis cases these included 1 infant with G2P[4], 1 with G12P[6] and 1 with G2P[14] gastroenteritis. Sensitivity analysis recategorizing these infants as secretors did not significantly alter the overall results for the relationship between genotype-specific susceptibility and HBGA status.

5.4.4 HBGA phenotype and odds of vaccine failure

To determine the relationship between HBGA phenotype and odds of clinical vaccine failure, 119 rotavirus gastroenteritis cases were compared to 119 age-matched community controls (one rotavirus gastroenteritis case had no saliva sample available for HBGA phenotyping). Non-rotavirus gastroenteritis controls were not included in HBGA analysis, due to potential bias

from other enteric infections associated with HBGA phenotype such as norovirus. The distribution of HBGA phenotype in this study population is summarized in Table 5-6.

Table 5-6: Distribution of secretor/Lewis negative phenotype in RV GE cases and controls

	Lewis positive	Lewis negative	Total
Non-secretor	28	19	47 (20%)
Secretor	155	36	191 (80%)
Total	183 (77%)	55 (23%)	238

The proportion of infants positive for both Le^a and Le^b was high: 91/238 (38%) were Le^{a+b+} partial secretors. Most of these infants were phenotypic secretors: 83/191 (43%) secretors and 8/47 (17%) non-secretors were Le^{a+b+}. Of these Le^{a+b+} non-secretor infants, five were confirmed as non-secretors by FUT2 genotyping, one had homozygous secretor genotype and one did not have blood available for genotyping. Unexpectedly, 12/47(26%) phenotypic non-secretors were Le^{a+b+}. Eight of these infants were confirmed as non-secretors by FUT2 genotyping, two had secretor genotype (one homozygous, one heterozygous), and two had no blood available for genotyping.

In contradiction to the original hypothesis, using matched conditional logistic regression, non-secretor phenotype was inversely associated with clinical vaccine failure: OR 0.39 (95%CI 0.20-0.75), p=0.005. Lewis negative phenotype was not significantly associated with odds of clinical vaccine failure: OR 0.70 (95%CI 0.37-1.3), p=0.27. Stratifying by secretor by Lewis phenotype did not improve predictive odds of vaccine failure (Table 5-7).

Table 5-7: RV vaccine failure by secretor/Lewis negative phenotype

HBGA Phenotype n=238	RV GE Cases (Vaccine Failures) n=119 OR (95%CI)		
Non-secretor n=47	n=14	0.4 (0.2-0.8)	p=0.005
<i>Lewis negative</i> n=19	n=8	-*	-
<i>Lewis positive</i> n=28	n=6	-	-
Secretor n=191	n=105	2.6 (1.3-5.0)	p=0.005
<i>Lewis negative</i> n=36	n=16	0.7 (0.3-1.6)	p=0.40
<i>Lewis positive</i> n=155	n=89	1.4 (0.6-3.4)	p=0.40

* - Insufficient pairs for matched analysis (conditional logistic regression)

There were differences in genotype distribution between secretors and non-secretors. In non-secretor infants, 7/13(54%) of RV infections where genotype was known were P[6] infections. In secretor infants only 26/101(26%) of RV infections where genotype was known were P[6] infections, and 5 of these were co-infections with P[8] or P[4]. The remaining RV infections where genotype was known were P[8] or P[4] infections.

FUT2 genotype was determined for 38/47 non-secretor infants: 7/38 (18%) of non-secretor infants tested (3 rotavirus gastroenteritis cases, 4 community controls) had a secretor genotype (3 homozygous and 4 heterozygous). Two of these infants were Le^{a-b+} and one was Le^{a+b+}. In a sensitivity analysis where all phenotypic non-secretor infants with secretor genotype were re-categorized as secretors, the negative association between non-secretor type and vaccine failure was unchanged: OR 0.36 (95%CI 0.17-0.74), p=0.005.

In a further sensitivity analysis, all Le^b positive infants who were known to be genotypic non-secretors were re-categorized as Le^b negative. The association between Lewis negative phenotype and vaccine failure remained non-significant: OR 0.61 (95%CI 0.33-1.1), p=0.11.

Additional analyses

There was no significant association between Le^{a+b-} phenotype and odds of vaccine failure: OR 1.3 (95%CI 0.34-4.7), p=0.74. However, this phenotype was uncommon in this population: only 9/238 (4%) infants were Le^{a+b-}. In secretor infants there was no significant association between ABO type and odds of vaccine failure by ABO type (Table 5-8).

Table 5-8: RV Vaccine Failure by ABO phenotype

ABO type n=191	RV GE Cases* (Vaccine Failures) n=105 OR (95%CI)		
A n=51	n=29	1.0 (0.51-2.0)	p=1.0
B n=51	n=26	0.75 (0.35-1.6)	p=0.18
O n=81	n=47	1.5 (0.76-2.8)	p=0.25
AB n=8	n=3	0.25 (0.03-2.2)	P=0.22

*Secretor infants only. Compared to secretor community controls

5.4.5 RV-specific IgA and odds of vaccine failure

RV-specific IgA was determined for 117 rotavirus gastroenteritis cases (vaccine failures), 119 community controls and 93 non-rotavirus gastroenteritis controls.

There was no significant difference in distribution of RV-specific IgA between non-rotavirus gastroenteritis and community controls (Table 5-9) therefore controls were grouped together for analysis to increase power.

Table 5-9: RV-specific IgA distribution in control groups

	Community controls	Non-RV GE controls	p*
% detectable RV-specific IgA	78/119, 66% (56-74%)	66/93, 71% (61-79%)	0.40
% RV-specific IgA >20U/mL	53/119, 45% (36-54%)	44/93, 47% (37-58%)	0.69
% RV-specific IgA >90U/mL	28/119, 24% (17-32%)	26/93, 28% (20-38%)	0.46

* χ^2 test

A lower proportion of RV1 vaccine failures had RV-specific IgA above the minimum detectable level compared to controls: 62/117 (53%, 44-62%) cases versus 144/212 (68%, 61-74%) controls had RV-specific IgA above the limit of detection (χ^2 p=0.007). Fewer RV1 vaccine failures were seropositive for RV-specific IgA: 28/117 (24%, 17-33%) had RV-specific IgA >20U/mL compared to 97/212 (46%, 39-53%) controls (χ^2 p<0.001). Very few RV1 vaccine failures had RV-specific IgA above the proposed “protective” level of 90U/mL: 9/117 (8%, 4-14%) compared to 54/212 (26%, 20-32%) controls (χ^2 p<0.001).

In those with detectable RV-specific IgA, the geometric mean concentration of RV-specific IgA was significantly lower in RV1 vaccine failures than in controls: 22.1 (95%CI 15.0-32.5) U/mL in cases versus 48.3 (95%CI 36.1-64.5) U/mL in controls (Mann Whitney p=0.0001) (Figure 5-5).

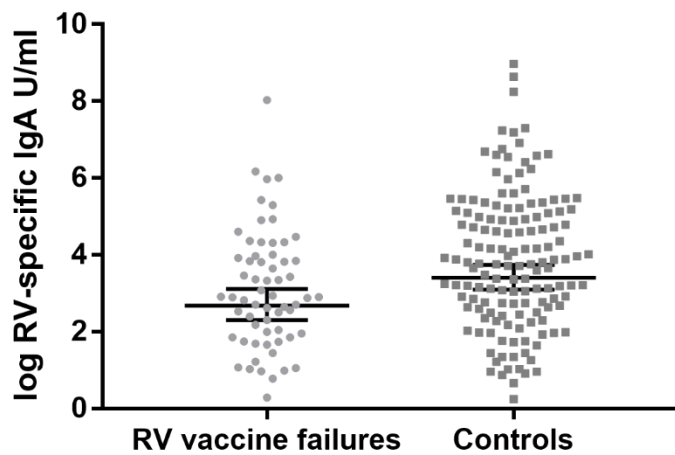


Figure 5-5: Comparison of RV-specific IgA between RV vaccine failures and controls

RV-specific IgA is log-transformed for easier visualization. Infants with undetectable RV-specific IgA are not included. Error bars show GMC with 95% CI.

Age-matched cases and controls were compared by conditional logistic regression. Lower RV-specific IgA was strongly associated with odds of RV1 vaccine failure. For every one log increase in RV-specific IgA, there was a 20% reduction in odds of RV vaccine failure: OR 0.80 (95%CI 0.70-0.90), $p < 0.001$. Seropositivity was associated with a 65% reduction in odds of vaccine failure: OR 0.35 (95%CI 0.20-0.61), $p < 0.001$. RV-specific IgA > 90 u/mL was associated with a 75% reduction in odds of vaccine failure: OR 0.25 (95%CI 0.12-0.55), $p = 0.001$.

5.4.6 RV-specific IgA response to natural infection in RV vaccine failures

To determine the RV-specific IgA response to natural infection in rotavirus vaccine failures, RV-specific IgA at presentation with rotavirus gastroenteritis was compared to convalescent RV-specific IgA. Paired presentation and convalescent samples were available for 60/120 (50%) rotavirus gastroenteritis cases. Median days after illness onset at convalescent sampling was 10 (IQR 9-12, range 6-19 days). Distribution of baseline RV-specific IgA was similar in those who with convalescent serology data available to those without (Table 5-10).

Table 5-10: Baseline RV-specific IgA in infants with and without convalescent serology

	Convalescent serology available	No convalescent serology	p
Detectable RV-specific IgA	31/60, 52% (39-64%)	31/57, 54% (41-67%)	0.77
RV-specific IgA >20U/mL	15/60, 25% (15-38%)	13/57, 23% (13-36%)	0.78
RV-specific IgA >90U/mL	4/60, 8% (3-19%)	4/57, 7% (3-18%)	0.79

Of infants with convalescent serology available, 31/60 (52%, 39-64%) had RV-specific IgA above the minimum detectable level at baseline; 15/60 (25%, 15-38%) were seropositive; and 5/60 (8%, 3-19%) had RV-specific IgA >90U/mL. In those with detectable RV-specific IgA, the baseline geometric mean concentration was 23.3 (95%CI 13.5-40) U/mL.

At follow-up, 59/60 (98%, 89-100%) infants had detectable RV-specific IgA, 57/60 (95%, 85-98%) had RV-specific IgA >20U/mL and 50/60 (83%, 71-91%) had RV-specific IgA>90U/mL. The follow-up geometric mean concentration was 389.5 (95% CI 252.4-601.1) U/mL, significantly higher than baseline (Wilcoxon matched-pairs signed rank test, $p<0.0001$) (Figure 5-6). The median convalescent fold-rise in RV-specific IgA concentration was 112.8 (IQR 19.1-380.6) fold.

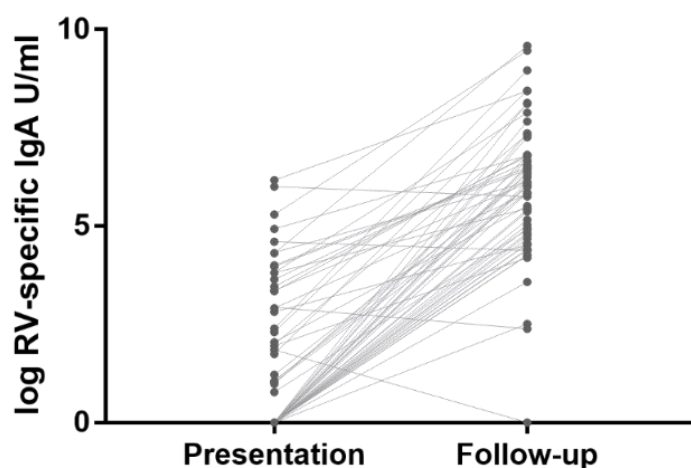


Figure 5-6: Convalescent rise in IgA GMC in RV Cases

One infant had an undetectable RV-specific IgA response at follow-up. This infant had a baseline RV-specific IgA of 6.4 U/mL, which is close to the limits of detection.

Of five infants who had a baseline RV-specific IgA >90U/mL, two showed a decline in RV-specific IgA at follow-up. Both infants reported symptoms for at least 5 days before initial presentation. The fold-rise in RV-specific IgA at follow-up for the remaining three infants was 6.5, 9.6 and 64.3-fold respectively, well below the median fold-rise for the population. Duration of symptoms in these infants prior to presentation was 1-3 days.

5.4.7 Demographic and socio-economic predictors of RV1 vaccine failure

Rotavirus gastroenteritis cases (vaccine failures) were compared to asymptomatic community controls by conditional logistic regression (Table 5-11). The median age at recruitment for community controls was 9.8 months (8.3- 11.1 months). There were no significant differences between cases and controls in sanitation and socioeconomic factors. History of a previous episode of diarrhoea, requiring attendance to the health centre increased the odds of rotavirus vaccine failure over 13-fold. HIV-exposure, male gender and low birth weight were not associated with increased odds of vaccine failure.

Anthropometric data were compromised by measurement errors, particularly in length measurement. Restrictions were applied in accordance with WHO guidelines to remove biologically implausible outliers (World Health Organisation, 1995). Length was replaced as missing if length for age score was below -4 or above +3. Length data (and consequently length for age and weight for length) was consequently lost for 24/120 cases and 24/120 controls. There were no implausible outliers in weight. Once implausible outliers were corrected and weight adjusted for dehydration status, there were no significant differences in weight/height based measures of nutritional status between cases and community controls. Lower MUAC (a measure of wasting) was associated with increased odds of vaccine failure. For every 1cm increase in MUAC, odds of vaccine failure were reduced by 40%. In infants over 6 months old, MUAC <11.5cm is a sign of severe wasting: 8/110 (7%, 4-14%) of infants with vaccine failure met this criteria, compared to none of 115 community controls (χ^2 p=0.003).

Table 5-11: Demographic and socio-economic factors: RV GE cases and community controls

Characteristic ^a	RV GE cases	Community Controls	OR	p ^b
<u>Infant Characteristics</u>				
Male	73, 61% (52-69%)	61, 51% (42-60%)	1.4 (0.9-2.3)	0.15
HIV-exposed ^c	17, 14%(9-22%)	19, 16%(10-24%)	0.9 (0.5-1.7)	0.73
Low birth weight (<2.5kg)	14/112 13% (8-20%)	12/118 9%(5-16%)	1.4 (0.6-3.4)	0.49
Prior diarrhoea (attended health centre)	60, 50% (41-59%)	10, 8% (5-15%)	13.5 (4.9-37.3)	<0.001
<u>Nutritional Status</u>				
Median weight for age z-score (IQR) ^d	-0.37 (-1.39-0.45)	-0.42(-1.0-0.36)	1.0 (0.8-1.3)	0.89
Median length for age z-score (IQR)	-0.68(-1.75-0.95)	-0.76 (-1.98- -0.06)	1.2 (1.0-1.4)	0.13
Median weight for length z- score (IQR) ^d	-0.62(-1.58-0.37)	-0.05(-1.32-0.88)	0.9 (0.7-1.1)	0.29
Median MUAC, cm (IQR)	13.2 (12.9-13.4)	13.8 (13.2-14.5)	0.6 (0.4-0.7)	<0.001
<u>Sanitation and socioeconomic factors</u>				
Median household size (IQR)	5 (4-6)	4 (3-6)	1.1 (0.9-1.4)	0.13
Non-piped water source	22, 19% (12-27%)	17, 14% (9-22%)	1.5 (0.7-3.1)	0.34
Time to access water				
<5minutes	27, 23% (16-31%)	20, 17% (11-25%)	ref	
5-30 minutes	52, 44% (35-53%)	52, 44% (36-54%)	0.8 (0.4-1.6)	0.54
>30 minutes	40, 34% (26-43%)	45, 38% (30-48%)	0.7 (0.3-1.5)	0.35
Pit-latrine type toilet	116, 97% (91-99%)	115, 96% (90-98%)	1.3 (0.3-4.7)	0.74
Electricity at home	61, 50% (42-59%)	55, 46% (37-55%)	1.2 (0.7-2.0)	0.76
One or more household members with salary	82, 68% (59-76%)	76, 63% (54-72%)	1.3 (0.7-2.1)	0.42
Household food insecurity	40, 33% (25-42%)	36, 30% (22-39%)	1.2 (0.7-2.0)	0.57
Median age of head of household in years (IQR)	30 (26-37)	30 (27-34)	1.0 (0.9-1.0)	0.30
Median years of maternal education (IQR)	8 (5-11)	9 (7-11)	0.9 (0.90-1.0)	0.30

a. All proportions reported as number (proportion, 95% confidence interval of proportion). Denominator for all proportions n=120 for both cases and controls unless stated otherwise. b. Bivariate analysis by conditional logistic regression c. 11/17 HIV exposed RV GE cases and 15/19 HIV exposed community controls had a negative HIV DNA PCR at 6 weeks old. One community control was known HIV infected and on ART. Status of remaining HIV exposed infants was unknown. Weight adjusted for dehydration status by adding 5% for some dehydration and 10% for severe dehydration.

Rotavirus gastroenteritis cases (vaccine failures) and non-rotavirus gastroenteritis controls were compared by conditional logistic regression for factors which may predict vaccine failure. There were no significant differences in infant characteristics, nutritional status or sanitation or socioeconomic measures between the two groups (Table 5-12).

Since there were no significant differences between rotavirus and non-rotavirus gastroenteritis cases, all gastroenteritis cases were combined and compared to community controls by conditional logistic regression. Prior history of diarrhoea and MUAC were significant predictors of gastroenteritis of any cause (Table 5-13).

Table 5-12: Demographic and socio-economic factors: RVGE cases and non-RV GE controls

Characteristic ^a	RV GE cases	Non-RV GE controls	OR (95%CI)	p ^b
<u>Infant Characteristics</u>				
Male	73, 61% (52-69%)	55, 58% (48-68%)	1.0 (0.6-1.8)	0.88
HIV-exposed ^c	17, 14%(9-22%)	15, 16% (10-25%)	1.2 (0.5-2.6)	0.68
Low birth weight <2.5kg	14/112 13%(8-20%)	11/88 13% (7-21%)	1.0 (0.4-2.5)	1.0
Prior diarrhoea (attended health centre)	60, 50% (41-59%)	39, 41% (31-51%)	1.2 (0.7-2.3)	0.45
<u>Nutritional status</u>				
Median weight for age z-score (IQR) ^d	-0.37 (-1.39-0.45)	-0.08 (-1.0-0.76)	0.9 (0.7-1.2)	0.43
Median height for age z-score (IQR)	-0.68(-1.75-0.95)	-0.82 (-1.80- 0.37)	1.1 (0.9-1.3)	0.43
Median weight for height z-score (IQR) ^d	-0.62(-1.58-0.37)	-0.22(-1.36-1.41)	0.9 (0.7-1.1)	0.25
Median MUAC, cm (IQR)	13.1 (12.4-14.0)	13.2 (12.5-14.0)	0.9 (0.7-1.3)	0.67
<u>Sanitation and socioeconomic factors</u>				
Median household size (IQR)	5 (3-6)	4 (3-6)	1.0 (0.8-1.3)	0.96
Non-piped water source	22, 19% (12-27%)	19/94, 20%(13-29%)	0.7 (0.3-1.6)	0.40
Time taken to access water				
<5minutes	27, 23% (16-31%)	23, 24% (17-34%)	ref	
5-30 minutes	52, 44% (35-53%)	42, 44% (34-55%)	1.1 (0.5-2.4)	0.83
>30 minutes	40, 34% (26-43%)	30, 32% (23-42%)	1.4 (0.5-3.4)	0.52
Pit-latrine type toilet	116, 97% (91-99%)	88, 93% (85-96%)	3.0 (0.6-14.9)	0.18
Electricity at home	61, 51% (42-60%)	54, 57% (47-67%)	0.8 (0.4-1.5)	0.41
One or more household member with salary	82, 68% (59-76%)	68, 72% (62-80%)	0.7 (0.4-1.3)	0.25
Household food insecurity	40, 33% (25-42%)	25, 26% (18-36%)	1.4 (0.8-2.5)	0.29
Median age of head of household (IQR)	30 (26-37)	32 (25-38)	1.0 (0.96-1.0)	0.86
Median years of maternal education (IQR)	8(5-11)	8(7-11)	1.0(0.90-1.1)	0.48

a. All proportions reported as number (proportion, 95% confidence interval of proportion). Denominator for all proportions n=120 for GE cases and n=95 for non-RV GE controls unless stated otherwise. b. Conditional logistic regression c. 11/17 HIV exposed RV GE cases and 7/15 HIV exposed non-RV GE controls had a negative HIV DNA PCR at 6 weeks. 1 non-RV GE control was known HIV infected and on ART. Status of remaining HIV exposed infants was unknown. d. Weight adjusted for dehydration status by adding 5% for some dehydration and 10% for severe dehydration.

Table 5-13 Demographic and socio-economic factors: all GE cases and community controls

Characteristic ^a	All GE cases	Community Controls	OR	p ^b
<u>Infant Characteristics</u>				
Male	128, 60% (53-66%)	61, 51% (42-60%)	1.4 (0.9-2.2)	0.12
HIV-exposed ^c	32, 15% (11-20%)	19, 16%(10-24%)	0.9 (0.5-1.7)	0.83
Low birth weight (<2.5kg)	25/200 13%(9-18%)	12/118 9%(5-16%)	1.4 (0.6-3.1)	0.46
Prior diarrhoea (attended health centre)	99, 46% (39-53%)	10, 8% (5-15%)	13.8 (5.5-34.6)	<0.001
<u>Nutritional Status</u>				
Median weight for age z-score (IQR) ^d	-0.23 (-1.31-0.61)	-0.42(-1.0-0.36)	1.0 (0.9-1.3)	0.62
Median length for age z-score (IQR)	-0.80(-1.75-0.69)	-0.76 (-1.98- -0.06)	1.1 (0.9-1.3)	0.29
Median weight for length z-score (IQR) ^d	-0.43(-1.54-1.02)	-0.05(-1.32-0.88)	1.0 (0.8-1.2)	0.76
Median MUAC, cm (IQR)	13.1 (12.5-14.0)	13.8 (13.2-14.5)	0.5 (0.4-0.7)	<0.001
<u>Sanitation and socioeconomic factors</u>				
Median household size (IQR)	5 (3-6)	4 (3-6)	1.1 (1.0-1.3)	0.12
Non-piped water source	41/213, 19%(14-25%)	17, 14% (9-22%)	1.6 (0.8-3.1)	0.21
Time to access water				
<5minutes	50, 23% (18-30%)	20, 17% (11-25%)	ref	
5-30 minutes	94, 44% (37-51%)	52, 44% (36-54%)	0.8 (0.4-1.5)	0.44
>30 minutes	70, 33% (27-39%)	45, 38% (30-48%)	0.8 (0.3-1.3)	0.23
Pit-latrline type toilet	204, 95% (91-97%)	115, 96% (90-98%)	0.8 (0.3-2.4)	0.72
Electricity at home	115, 54% (47-60%)	55, 46% (37-55%)	1.4 (0.9-2.2)	0.18
One or more household members with salary	150, 70% (63-76%)	76, 63% (54-72%)	1.4 (0.9-2.3)	0.16
Household food insecurity	65, 30% (24-37%)	36, 30% (22-39%)	1.0 (0.6-1.6)	0.97
Median age of head of household (IQR)	32 (26-38)	30 (27-34)	1.0 (0.9-1.0)	0.23
Median years of maternal education(IQR)	8 (6-11)	9 (7-11)	1.0 (0.9-1.0)	0.45

a. All proportions reported as number (proportion, 95% confidence interval of proportion). Denominator for all proportions n=215 for GE cases and n=120 for community controls unless stated otherwise. b. Conditional logistic regression c. 17/32 HIV exposed GE cases and 15/19 HIV exposed community controls had a negative HIV DNA PCR at 6 weeks. 1 non-RV GE case and one community control were known HIV infected and on ART. Status of remaining HIV exposed infants was unknown. d. Weight adjusted for dehydration status by adding 5% for some dehydration and 10% for severe dehydration.

5.4.8 Multivariable model.

Factors identified as the most significant predictors of vaccine failure ($p < 0.10$) were combined in a multivariable conditional logistic regression model. Where variables were co-linear, only the variable with the lowest p value was tested initially, and other significant factors tested separately. Manual stepwise backwards elimination was used. At each step, the variable with the highest p value was eliminated until only variables with p values < 0.10 remained.

In multivariable analysis comparing community controls and RV vaccine failure cases, MUAC, previous attendance at a health centre for diarrhoea and RV-specific IgA remained significant predictors of RV vaccine failure. Lewis positive secretor phenotype, the HBGA phenotype most significantly associated with vaccine failure, did not retain significance in multivariable analysis.

Of note, there was some collinearity between the predictor “previous attendance at health centre for diarrhoea” and MUAC. For every 1 cm increase in MUAC, the odds of previous attendance at a health centre for diarrhoea decreased by around a third: OR 0.67 (95%CI 0.55-0.82). Non-secretor phenotype and prior history of diarrhoea were also weakly inversely correlated – non-secretor phenotype reduced the odds of previous attendance at a health centre for diarrhoea by 50%: OR 0.50 (95%CI 0.27-0.93). There was no evidence of collinearity between any of the other predictive variables.

Various iterations of the model were determined including and excluding collinear variables. The final model (Table 5-14) had the best fit as compared by Akaike Information Criterion (AIC). Only prior diarrhoea, MUAC and log-RV specific IgA remained significant predictors of clinical vaccine failure in the final model.

Table 5-14: Multivariable model of predictors of vaccine failure

Characteristic	Bivariable analysis OR (95%CI)	Multivariable analysis Adjusted OR (95%CI)
Prior diarrhoea (attendance at health centre)	13.5(4.9-37.3) $p < 0.001$	18.9 (5.1-70.0) $p < 0.001$
MUAC (cm)	0.55(0.42-0.73) $p < 0.001$	0.64 (0.44-0.91) $p = 0.01$
Log RV-specific IgA	0.80 (0.70-0.92) $p = 0.002$	0.76 (0.62-0.93) $p = 0.008$
Non-secretor phenotype	0.39 (0.20-0.75) $p = 0.005$	NS

5.5 DISCUSSION

Rotavirus infection in a Malawian population with established high vaccine coverage

Rotavirus surveillance in Blantyre, Malawi has been established for over twenty years, providing an overview of shifting genotype distribution over time and an opportunity to observe the impact of RV1 vaccine introduction. Vaccine introduction has seen a 54% decline in rotavirus hospitalization incidence in Malawi in infants under 12 months old, with incidence from January to June 2015 estimated at 123/100,000 infant population (Bar-Zeev et al., 2016). The mean age of presentation with rotavirus gastroenteritis has increased from 9.3 (SD 5.2) months pre-vaccine to 11.8 (SD 5.8) months post-vaccine introduction, with just under half of all cases now occurring in children over 12 months old (Bar-Zeev et al., 2016). To explore predictors of primary and early secondary vaccine failure, the focus of my study was vaccine failure in the first year of life. Due to the changing epidemiology of rotavirus gastroenteritis post vaccine introduction, one limitation of this approach was a lower than expected numbers of cases, compounded by a high number of families who declined to participate. The number of severe rotavirus gastroenteritis cases recruited was just under the target sample size, but still with sufficient power to examine the primary outcome measures. It should be remembered that severe rotavirus gastroenteritis remains associated with significant mortality – 2.5% in the study population -and the welcome decline in incidence post-vaccine introduction does not diminish the importance of reducing the burden of rotavirus disease further.

In common with many other sub-Saharan African countries, rotavirus genotypic diversity in Malawi is high, and there is a high proportion of mixed infections. P[6] infections are much more frequently identified in Africa, with the commonest P[6] strains accounting for around 24% of all rotavirus infections, compared to <6% elsewhere in the world (Todd et al., 2010). The high proportion of infections with P[6] strains (29%) in the study population is in keeping with this wider African data. The possibility that this higher proportion of P[6] may relate to population prevalence of Lewis negative phenotype, which may predispose to P[6] infection, is discussed further below.

In the study population, G1P[8] was the predominant strain, identified in 32% of severe rotavirus gastroenteritis cases. This is a higher proportion than that observed just prior to vaccine introduction in 2012, when the proportion of rotavirus gastroenteritis cases due to G1P[8] was 16% (Jere et al., 2018). Whether the emergence of novel G1P[8] strains represents natural oscillation or selective vaccine pressure remains unclear.

Asymptomatic rotavirus infection was common – rotavirus was detected by qRT-PCR in 64% of community controls. This was a higher prevalence of asymptomatic infection than reported in previous studies in Malawi (31%), Ecuador (12%) and England (24%)(Phillips et al., 2010, Bennett et al., 2015, Lopman et al., 2013). However, these previous studies included children aged up to 5 years, and rates of asymptomatic infection may be higher in younger infants. Two studies in Nigeria prior to vaccine introduction detected rotavirus by EIA in 15-19% and 30% of infants respectively (Abiodun et al., 1985, Omoigberale et al., 1996). Given the lower sensitivity of EIA, and the low viral load of most asymptomatic infections, these rates are likely comparable, if not higher, than those in the study population. Asymptomatic infection was also common in Vellore in an unvaccinated cohort followed from birth to three years: incidence rate of asymptomatic rotavirus infection was 0.75 infections per child year, compared to 0.29 symptomatic infections per child year (Paul et al., 2014).

In the study population, the two most common genotypes in asymptomatic infection mirrored those causing rotavirus gastroenteritis. G12P[8] infection was the third most common. Analysis of genotype distribution was limited by low-viral load in most asymptomatic infections. There are few published data on the genotype distribution of asymptomatic rotavirus infection outwith the neonatal period in Malawi or any other setting.

My data on asymptomatic infection must be considered within the limitations of a cross-sectional case:control design. Detection of rotavirus might represent convalescent shedding following prior gastroenteritis or detection of an evolving infection in the incubation phase rather than true asymptomatic infection (Levine and Robins-Browne, 2012). Although I excluded infants who had gastroenteritis symptoms within the previous week, this time period may have been too short. Only 10/120(8%) controls reported any prior attendance at a health

centre for diarrhoea, but we did not record the timing of these attendances in relation to recruitment. A longitudinal cohort design would be better suited to exploring the incidence, genotype and predictors of asymptomatic rotavirus in Malawian infants.

HBGA phenotype and genotype-specific susceptibility to rotavirus

My study confirmed that HBGA secretor/Lewis phenotype is associated with genotype-specific rotavirus gastroenteritis.

Secretor phenotype was associated with increased odds of P[8] rotavirus gastroenteritis. This was driven predominantly by a very strong association between G1P[8] gastroenteritis and secretor phenotype, where all 44 G1P[8] gastroenteritis cases were secretors. A corresponding, but weaker, inverse association was observed for Lewis negative infants. The association with secretor phenotype is consistent with observational and case:control studies of rotavirus gastroenteritis in Burkina Faso, Nicaragua, France, Vietnam, Tunisia, USA and China (Nordgren et al., 2014, Imbert-Marcille et al., 2014, Trang et al., 2014, Ayouni et al., 2015, Payne et al., 2015, Zhang et al., 2016, Sun et al., 2016). Not all of these studies determined Lewis phenotype – those which did observed a similar trend in positive association with Le^{b+} phenotype (Nordgren et al., 2014, Ayouni et al., 2015, Trang et al., 2014, Zhang et al., 2016, Sun et al., 2016).

I did not determine any association between secretor or Lewis phenotype and asymptomatic P[8] infection. Although numbers were small, six of seventeen asymptomatic P[8] infections, including three G1P[8] asymptomatic infections were in non-secretors. No prior published study has included children with asymptomatic rotavirus infection. I believe these data provide proof of principle that HBGA phenotype may not determine susceptibility to asymptomatic infection. This is consistent with my hypothesis, based on observed shedding of G1P[8] vaccine in non-secretors in Chapter 3, that resistance of non-secretors to P[8] infection is partial, rather than absolute. This is supported by serological surveys in Sweden and China, which showed that while P[8]-neutralizing antibody (Sweden) and P[8]-specific IgG (China) titres were reduced in non-secretors, they were not absent (Gunaydin et al., 2016, Zhang et al., 2016). This might suggest an immunological response consistent with low-viral load infection, which is more likely

to be asymptomatic. Further studies with larger numbers of asymptomatic rotavirus infections of diverse genotypes are needed to prove this hypothesis.

Lack of a strong association between HBGA phenotype and asymptomatic infection may explain the overall lack of an association between P[8] infection and secretor/Lewis phenotype in this population. It is also possible that the association between HBGA phenotype and susceptibility might depend on both G and P Type. In the study population, G1P[8] infections were most strongly associated with secretor phenotype. There were 10 non-G1 P[8] infections in this population: 4 G12P[8] (all asymptomatic), 4 G2P[8] (3 symptomatic) and 2 mixed G2P[8]/P[6] (1 symptomatic) infections. Numbers were too small for sub-group analysis by HBGA phenotype, but four (2 G12P[8], 2 G2P[8]) of the ten were in non-secretors.

Of note, a recently published cohort study from Bangladesh similarly found no association between secretor phenotype and risk of P[8] infection (Lee et al., 2018). This study included rotavirus diarrhoea of any severity, and the authors reflected that non-secretor status could have limited the severity of P[8] rotavirus infection, and the higher proportion of mild cases in their surveillance cohort could explain the lack of association with secretor phenotype. Also of note, 90% of P[8] infections in non-secretors were G9P[8] infections. This would support my hypothesis that non-secretors are less protected against non-G1 P[8] strains.

Most previous studies reporting the association of HBGA phenotype with rotavirus gastroenteritis have reported results only by P Type, which makes comparison difficult. Payne et al. in the USA found all 136 G12P[8] gastroenteritis cases were in secretors (Payne et al., 2015). Further data are required to explore this possibility further. A retrospective analysis of G type in all currently published HBGA/rotavirus studies would be a useful start.

I also determined that P[4] infection was associated with Lewis positive phenotype. Although a weak association with secretor phenotype was also noted, this appeared to be driven by the overlap with Lewis positive phenotype. This finding is supported by *in vitro* binding studies. Huang et al. (2012) and Ma et al. (2015) both demonstrated that P[4] strains bound Le^b and H Type 1 antigens. Zhang et al. (2016) demonstrated that P[4] strains showed binding to saliva of Le^{b+} but not Le^{b-} secretors. To date, five studies have clearly reported P[4] infection by

Le^b phenotype, although numbers of P[4] infection in some studies were small. Four studies in Burkina Faso and Nicaragua, Tunisia, China and Bangladesh collectively reported a total of 52 P[4] infections, all in Le^{b+} secretors (Nordgren et al., 2014, Ayouni et al., 2015, Zhang et al., 2016, Lee et al., 2018). In contrast, another Chinese study reported 22 P[4] infections, all in secretors or partial secretors, but only 11/22 (50%) were Le^{b+}, compared to a population prevalence of Le^{b+} phenotype in rotavirus negative controls of 71%. However, the distribution of Lewis phenotype in Asia differs from other populations, and 21/22 P[4] infections were in Le^{y+} individuals (Sun et al., 2016). Le^y is a structural analogue of Le^b and may show similar binding patterns.

My finding of an association between P[4] infection and Lewis positive phenotype is also consistent with results from a serological survey in China, where rates of detectable P[4] specific IgG were significantly higher in Le^{b+} compared to Le^{a+b-} healthy volunteers (73% vs 39%, $p < 0.001$) (Zhang et al., 2016).

Finally, I determined an association between Lewis negative phenotype and P[6] infection. Lewis negative phenotype was similarly associated with increased odds of P[6] infection in Burkina Faso: OR 5.5 (95%CI 2.3-13.2, $p < 0.001$) (Nordgren et al., 2014) and in Bangladesh: RR 34.3 (95%CI 4.20-281), $p < 0.001$ (Lee et al., 2018). The association between Lewis negative phenotype and P[6] infection is consistent with results from binding studies. Huang et al. (2012) demonstrated that VP8* proteins of P[6] bound to H Type 1 antigen, but not Le^b, and in salivary binding studies P[6] only bound saliva samples of Le^b negative individuals. Similarly, Ma et al. (2015) showed only weak binding of P[6] to Le^b in both oligosaccharide binding assays and saliva and no binding to Le^a or Le^x. An explanation for this pattern may lie in findings of Liu et al. (2016), who identified that Lewis epitope blocked binding of P[6] to H Type 1 antigen. This would explain a higher risk in ABH secretors who were Lewis negative, while Lewis positive secretors would be relatively protected. This was evident in the stratified analysis in the study data.

The association between Lewis negative phenotype and increased susceptibility to P[6] rotavirus infection could explain the higher proportion of P[6] infection observed in sub-

Saharan Africa. The prevalence of Lewis negative phenotype is higher in Africa (32% in Burkina Faso, 20% in the study population) than in most European populations (7-10%). This, combined with the relative protective association between Lewis negative phenotype and P[8] and P[4] infection, could offer a selective advantage to P[6] infection in this setting. The prevalence of HBGA phenotypes within a population could therefore be important determinants of rotavirus evolution and epidemiology. The possibility that HBGA phenotype could contribute to human virus co-evolution should also be explored.

HBGA phenotype as a predictor of vaccine failure

Contrary to my original hypothesis, non-secretor phenotype was associated with a 60% decrease in the odds of vaccine failure.

In my original hypothesis, I proposed that non-secretor phenotype/Lewis negative phenotype would be associated with reduced RV1 vaccine response, and therefore reduced clinical protection, due to inherent resistance to P[8] rotavirus infection. The overall effect of HBGA phenotype on clinical vaccine failure would depend on the balance between protective effects against disease, and inhibitory effects on vaccine response.

In Chapters 3 and 4, I found limited evidence that HBGA phenotype predicted vaccine virus replication, only weak evidence increased vaccine virus replication in secretors following the first dose of immunisation, but no association overall. Vaccine virus replication occurred in a large proportion of non-secretor and Lewis negative infants, and HBGA phenotype did not predict antibody response. From these data, I revised my hypothesis to propose that HBGA-related resistance to P[8] infection was partial, rather than absolute. Partial resistance might allow P[8] infection in non-secretor/Lewis negative infants, but limit virus replication. Since lower viral load infections are less severe, this would protect these infants against P[8] rotavirus gastroenteritis, but not asymptomatic infection. Asymptomatic infection could potentially allow further boosting of protective immunity (Lewnard et al., 2017).

Non-secretor phenotype was associated with protection against P[8] gastroenteritis, particularly G1P[8] gastroenteritis, the commonest cause of rotavirus gastroenteritis in this population. In addition, non-secretor phenotype was associated with protection against P[4]

infection, the second most common gastroenteritis strain. In this context, any inhibitory effect of non-secretor phenotype on rotavirus vaccine response is clearly outweighed by protection against the commonest strains associated with rotavirus vaccine failure.

The effect of Lewis phenotype on vaccine failure was more complex. Lewis negative phenotype is associated with decreased risk of both P[8] and P[4] infection, but increased risk of P[6] infection. These opposing effects meant there was no association between Lewis phenotype and overall odds of vaccine failure. It might be predicted that in other settings with a lower proportion of P[6] infection, and higher proportion of P[8]/P[4], Lewis positive phenotype might be associated with an overall increased risk of clinical vaccine failure.

In a large US surveillance study, Payne et al. (2015) also determined that non-secretors were at greatly reduced risk of vaccine failure: 1/110 (1%) rotavirus vaccine failures were non-secretors, compared to 199/1104 (18%) rotavirus negative gastroenteritis controls (OR 0.04 (95%CI 0.006-0.30), $p=0.001$). However, in this study, 91% of gastroenteritis cases were due to P[8] infection.

In Bangladesh, non-secretor phenotype was not significantly associated with rotavirus vaccine failure: RR 0.75 (95%CI 0.41-1.48), $p=0.49$), however the number of non-P[8]/P[4] infections in this cohort was small(Lee et al., 2018). The authors commented that further studies in settings of greater genotypic diversity were required.

Malawi has a proportion of non-secretors comparable to most populations, a high proportion of both Lewis negative infants and a diverse rotavirus genotype distribution, where non-P[8] rotavirus infections are common. If the hypothesis that HBGA phenotype contributes to reduced vaccine efficacy in low-income settings was to be demonstrated anywhere, this would be an ideal population.

Instead, I found evidence that non-secretor status is associated with reduced risk of clinical vaccine failure, with no overall effect of Lewis phenotype. In this Malawian population, 105/119(88%) of vaccine failures were in secretors and 95/119(80%) were Lewis positive. In non-secretors with vaccine failure, 2/14 (14%) had P[8] rotavirus gastroenteritis. The initial HBGA hypothesis would not explain vaccine failure in any of these infants. Clearly other causes of vaccine failure are more important in this population.

My findings would suggest that non-P[8] based vaccines would be unlikely to significantly improve vaccine efficacy in Malawi, at least not on the basis of the HBGA hypothesis. On the contrary, given the association between Lewis positive phenotype and reduced risk of P[6] infection, we might hypothesize that P[6]-based vaccines, such as the neonatal vaccine RV3-BB, might perform less well in Lewis positive infants, who comprise over 75% of the population. Against this hypothesis, RV3-BB has shown good immunogenicity in other settings (Bines et al., 2015). It may be that, similar to P[8] vaccines, any reduction in virus replication may be minimal, and may not preclude an adequate immune response. Studies to confirm this would be reassuring.

Low RV-specific IgA is strongly associated with reduced clinical protection

Lower RV-specific IgA at time of presentation was strongly associated with increased odds of RV1 clinical vaccine failure. This is consistent with evidence that RV-specific IgA is the best available surrogate of protection at population level (Patel et al., 2013). Seropositivity (RV-specific IgA > 20 U/mL) was associated with a 64% reduction in vaccine failure (OR 0.36 (95%CI 0.21-0.62), $p < 0.001$). This is highly consistent with the estimated protective effect of seropositivity in a post-hoc analysis of data from the Malawi/South Africa rotavirus vaccine trial, where seropositivity post-vaccine was associated with similarly reduced odds of subsequent severe rotavirus gastroenteritis (OR 0.39 (95%CI 0.29-0.52), $p < 0.001$) (Cheuvart et al., 2014). The proposed protective level (Patel et al., 2013) also proved highly predictive: RV-specific IgA > 90 U/mL was associated with a 75% reduction in odds of vaccine failure: OR 0.25 (95%CI 0.11-0.53), $p < 0.001$). Although 9/114 (8%, 4-14%) of infants with clinical vaccine failure had RV-specific IgA at presentation above this level, in some infants this may have been due to sampling when IgA was already rising in response to acute infection.

In contrast, data from the two largest cohort studies of natural rotavirus infection and immunity in Mexico and Vellore, India have not provided consistent evidence of a strong protective effect of RV-specific IgA against rotavirus gastroenteritis (Velazquez et al., 1996, Premkumar et al., 2014). In a paired reanalysis of both studies, Lewnard et al. (2017) found that, when adjusted for age, each 2-fold increase in IgA was associated with a decrease in rate

of acquired rotavirus infection of 13% in Mexico, and 8% in Vellore, but a protective threshold could not be established. In addition, while in Mexico increasing IgA was associated with decreased risk of rotavirus gastroenteritis, in Vellore no association was found between serum RV-specific IgA and risk of symptomatic rotavirus disease. One limitation of these studies was that RV-specific IgA was measured at 4-6 monthly intervals, and not at the time of infection or disease. The studies also looked at any rotavirus diarrhoea, rather than severe disease.

Further data from diverse populations are required to determine whether any level of RV-specific IgA can be considered protective, or at least highly predictive of protection against clinical vaccine failure. Determining a reliable protective threshold would greatly assist the evaluation of new vaccines or vaccine strategies such as booster dosing.

RV-specific IgA response to natural infection in RV vaccine failure

I confirmed that in this population, infants with rotavirus vaccine failure show a robust RV-specific IgA response to natural infection. Almost all infants demonstrated a significant rise in RV-specific IgA titres within 10 days of symptom onset. Of those who did not, most had high titres at baseline, suggesting that immune response was adequate, but sampling occurred late in the course of infection. Only one infant showed no evidence of RV-specific IgA response. This infant could possibly have selective IgA deficiency, a common primary immunodeficiency.

The case-control study design did not allow comparison of response to natural infection to initial vaccine response, however we can still reflect on the possible implications of this finding.

RV-specific IgA in most infants was low at time of vaccine failure. This suggests either inadequate vaccine response (primary vaccine failure), or adequate or sub-optimal vaccine response with waning immunity over time (secondary vaccine failure).

In primary vaccine failure, a subsequent robust response to natural infection suggests that whatever factor limited immune response to the initial vaccine was either vaccine-specific or transient. For example, vaccine-specific effects might occur where the gut environment prevented adequate replication of a low-infective load attenuated vaccine virus, but allowed adequate replication of a high-infective load wild-type virus with subsequent mucosal immune

response. Transient inhibitory factors present at time of immunization but not at time of natural infection would include maternal antibody, concurrent OPV immunization and co-infection.

Secondary vaccine failure might occur due to gradual waning of immunity with increasing age, or the development of a condition increasing susceptibility to disease or associated with acquired immunodeficiency. The development of a condition increasing susceptibility to disease would not explain the lower baseline IgA observed in most vaccine failures. Acquired immunodeficiency is less likely given the robust response to infection seen in most children. Waning of immunity following an initially adequate vaccine response appears most likely as the cause of secondary vaccine failure.

The proportion of infants with primary or secondary vaccine failure cannot be determined from a case:control design. Infants with primary vaccine failure due to transient inhibitory factors, and infants with secondary vaccine failure due to waning immunity might benefit from a booster dose of rotavirus vaccine in later infancy. For logistic reasons, a booster dose at 9 months, to coincide with the existing scheduled vaccination visit for measles vaccine, might be most cost-effective. A “proof of concept” randomized trial in Bangladesh determined the immunogenicity of a booster rotavirus vaccine given concurrently with measles-rubella vaccine in 9 month old infants who had received two previous doses of RV1 at 6 and 10 weeks of age (Zaman et al., 2016). A modest rise in RV-specific IgA was seen overall: seropositivity rates increased from 52.7% pre-booster to 69.6%. However, the improvement was more significant in infants who were seronegative at 9 months, 43.6% of whom seroconverted post booster. My study data would suggest that these seronegative infants are those at highest risk of vaccine failure, and potentially most likely to benefit from booster dosing. However, since the median age of rotavirus vaccine failure in the case-control study was 9 (IQR 7.6-10.6) months, a booster dose at this stage might be too late to protect a significant proportion of infants.

A cohort study determining the dynamics of IgA response from vaccination throughout the first year of life could help determine optimal scheduling and the utility of booster dosing.

Nutritional status and increased risk of gastroenteritis

Lower mid-upper arm circumference (MUAC), a measure of wasting, was associated with increased odds of vaccine failure. For every 1cm increase in MUAC, odds of vaccine failure were reduced by 40%. Compared to community controls, infants with clinical vaccine failure were much more likely to have a MUAC <11.5cm, a sign of severe wasting in infants over 6 months old. However, MUAC was similarly associated with increased odds of non-rotavirus gastroenteritis, and did not predict odds of clinical vaccine failure when compared to non-rotavirus gastroenteritis controls. This suggests that lower MUAC is associated with increased risk of gastroenteritis of any cause, rather than being a specific predictor of rotavirus vaccine failure. There was no association found between any other measure of nutritional status and risk of either clinical vaccine failure or diarrhoea of any cause.

As discussed in Chapter 1, there is limited and contradictory evidence that malnutrition is associated with reduced rotavirus vaccine efficacy. In sub-group analyses of malnourished children, Perez-Schael et al. (2007) in Latin America found no evidence of reduced rotavirus vaccine efficacy, whereas Gastanaduy et al. (2016b) in Botswana reported a borderline significant reduction. Neither study was adequately powered for these sub-group analyses, with small numbers of malnourished children in both populations. Both studies used nutritional status at time of immunization for comparison. As noted in Chapters 3 and 4, I found no evidence that poor nutritional status at baseline was associated with reduced vaccine response. It is more likely that any association between malnutrition and reduced clinical protection is due to secondary vaccine failure, mediated through a general increased risk of susceptibility to severe diarrhoeal disease.

The relationship between diarrhoea and other infections and malnutrition is complex and bidirectional. There is a long history of literature supporting the hypothesis that recurrent and persistent diarrhoea contributes to poor growth and malnutrition, via several mechanisms including worsening enteropathy, increased catabolism, decreased dietary intake and nutrient sequestration (Brown, 2003, Schlaudecker et al., 2011). Poor nutrition may also increase risk of diarrhoea, through reduced mucosal immunity, impaired gut integrity, and micronutrient

deficiency, however the epidemiological evidence to support a causal association in this direction has been controversial (Brown, 2003). The effect of poor nutrition on diarrhoeal risk may be more relevant to some pathogens than others. For example, in a cohort study of children aged 2-5 years in Bangladesh, malnutrition at enrolment was associated with increased subsequent risk of diarrhoea secondary to enterotoxigenic *Escherichia coli*, *Cryptosporidium* sp., and *Entamoeba histolytica* but was not associated with increased risk of diarrhoea due to viruses (Mondal et al., 2009). Further to this finding, Das et al. (2017) in an analysis of twenty years surveillance data in urban Dhaka, Bangladesh, found that after adjusting for climate, socio-demographic factors and sanitation practices, underweight or wasted children were relatively protected from rotavirus disease, compared to well-nourished children. This inverse association between malnutrition and symptomatic rotavirus was also seen in a separate cohort study of infants in Dhaka (Verkerke et al., 2016). The proposed mechanism for this protective effect was via malnutrition associated enteropathy –causing damage to the intestinal epithelium and reducing the efficiency of rotavirus attachment and replication. The finding in Chapter 3 that lower MUAC is associated with reduced likelihood of rotavirus vaccine virus shedding would be consistent with this hypothesis.

If poor nutritional status is associated with protection against rotavirus, why did we not identify this association in this population? And what might explain the increased odds of rotavirus gastroenteritis and gastroenteritis of any cause in infants with lower MUAC?

Firstly, exploration of nutritional status a predictor of vaccine failure was a secondary end-point. My study is limited by small numbers, and power further reduced by errors in length measurement which resulted in the exclusion of a significant proportion of infants. These errors occurred despite detailed standard operating procedures and repeated training of research nurses in anthropometric measurement, and reflect the difficulty of obtaining accurate length measurement in young infants. I also adjusted weight to account for individual hydration status in gastroenteritis cases. I believe this analytic approach was important to avoid bias, but acknowledge that effects of dehydration on weight may have been overestimated, masking an association between vaccine failure and weight-based anthropometric measures. In hindsight, a protocol change to measure weight on discharge rather than admission could have reduced this

problem. Inadequate power in this sub-group analysis could explain failure to identify any association, positive or negative, between weight and length based anthropometric measures and odds of rotavirus gastroenteritis.

Secondly, my analysis is limited to severe gastroenteritis cases, including a large proportion of children who were hospitalized. Infants with severe malnutrition have less clinical reserve and therefore may become more unwell with diarrhoeal illness. Regardless of diarrhoeal symptoms, they are more likely to be admitted to hospital for nutritional management. This could have resulted in a recruitment bias of malnourished children in hospitalized cases compared to community controls.

Finally, the study was limited by a cross-sectional case:control design and cannot therefore determine a direction in causality in the association between lower MUAC and diarrhoeal disease. This is particularly important given the observed association between prior diarrhoea and MUAC, where for every 1cm increase in MUAC the odds of prior attendance at a health centre for diarrhoea decreased by around one-third. As discussed above, since there is evidence from several cohort studies that recurrent diarrhoeal disease is associated with poor growth, this could be the direction of causality, or there could be bidirectional effects.

A large, multi-country cohort study would be required to determine conclusively the direction of association between nutritional status and secondary rotavirus vaccine failure.

Prior diarrhoea as a predictor of vaccine failure

Unexpectedly, the strongest predictor of severe rotavirus gastroenteritis in vaccinated infants was a prior episode of diarrhoea requiring attendance at a health centre. Half of all infants with clinical vaccine failure reported a prior history of diarrhoea, compared to only 8% of age-matched community controls. Prior history of diarrhoea was reported by families and not based on health records, therefore may have been subject to recall bias. As diagnostic stool testing is not routinely available in Malawian health centres there was no way to establish the aetiology of prior diarrhoeal episodes.

If the acquisition of rotavirus infection is similar in infants with vaccine failure to the natural history of rotavirus in unvaccinated infants, then these prior diarrhoeal episodes may also have been due to rotavirus. In a further analysis of the Vellore birth cohort, Lewnard et al. (2017) determined that infants who developed gastroenteritis with a prior rotavirus infection had a 74% higher risk that their next rotavirus infection would also be symptomatic, compared to infants with an asymptomatic prior infection. This effect was independent of age and rotavirus-specific antibody levels. The authors speculated that this association might reflect a subset of infants with a particularly high susceptibility to symptomatic disease. As discussed above, high susceptibility to symptomatic disease will increase the risk of clinical vaccine failure. Clinical vaccine failure could potentially present as multiple symptomatic rotavirus infections until natural immunity is finally acquired. In this respect, in a case:control design, a history of prior diarrhoea may not represent a predictor of subsequent vaccine failure, but a proxy indicator of earlier vaccine failure.

Alternatively, prior diarrhoeal episodes may have been due to other enteropathogens and not rotavirus. In support of this alternative is the finding that a prior history of diarrhoea was also strongly associated with odds of non-rotavirus gastroenteritis. This could reflect a subset of infants at a higher general risk of diarrhoeal disease, and this may overlap with risk of rotavirus clinical vaccine failure. This could be secondary to factors associated with increased exposure to enteric pathogens due to overcrowding or poor sanitation, or increased clinical vulnerability such as poor gut health.

It is also possible that prior diarrhoeal disease directly increases the risk of subsequent gastroenteritis. For example, prior enteric infection might cause gut inflammation which could result in transient mucosal immune dysfunction, or transient changes in the microbiome, which in subsequent infection could facilitate virus replication leading to symptomatic disease. This would be consistent with recently published data from the MAL-ED study, a large multi-site birth cohort study. In this cohort, high enteric pathogen detection in stool was associated with increased risk of rotavirus diarrhoea (Mohan et al., 2017). In Malawi, a large cohort study approach looking at in detail at risk factors for both rotavirus and non-rotavirus gastroenteritis

in infants would be required to confirm the sequence of enteric infection and whether this relates to subsequent risk of diarrhoeal disease.

Given the clear indications that a subset of children are at higher diarrhoeal risk, it is perhaps surprising that no other proxy markers of overcrowding, sanitation or poverty predicted either rotavirus vaccine failure or gastroenteritis of any cause. As discussed previously in Chapters 3 and 4, this may be due to the difficulties of adequately stratifying risk and identifying appropriate proxy measures in this uniformly poor population. For example, I measured total number of individuals per household as a proxy for overcrowding, and found no significant association. The MAL-ED study used the definition “more than 2 people per room” and identified a weak association with increased risk of rotavirus diarrhoea (OR 1.4 (95%CI 1.0-1.8), $p=0.01$). However, consistent with my data, the MAL-ED study in one of the largest cohorts to date found no association between any other measure of poverty, maternal education, sanitation or socio-economic status and rotavirus diarrhoea (Mohan et al., 2017) in either vaccinated or unvaccinated populations. The interactions between enteric infection, malnutrition and socio-economic factors are complex and a traditional variable selection approach to identify risk factors may be overly simplistic. In an interesting alternative approach, the PROVIDE study, a large birth cohort in Bangladesh aiming to identify predictors of oral vaccine performance, used clustering analysis to identify relationships between predictor variables (biomarkers of vaccine failure)(Naylor et al., 2015). In early life, diarrhoeal disease clustered with systemic inflammation, but not with markers of environmental enteropathy, which instead clustered with subclinical enteropathogen presence. Subclinical enteric infection, but not diarrhoea, also clustered with poor sanitation and micronutrient deficiency. Socioeconomic factors such as maternal education clustered with weight/height based measures of nutritional status, but not with diarrhoea or subclinical enteric infection. The authors of PROVIDE propose different pathways to vaccine failure based on these three clusters. It seems plausible that these, or similar, pathways could correspond to different clinical vaccine failure phenotypes. For example, the child with rotavirus vaccine failure in the context of recurrent diarrhoea and systemic inflammation may follow a different mechanistic pathway to vaccine failure than the child with chronic subclinical infection and micronutrient

deficiency, or the otherwise thriving child in an overcrowded household with high force of infection. The presence and proportion of different clinical vaccine failure phenotypes within a population could determine the overall predictors of vaccine failure specific to that population. Different predictors could be important to different children, potentially bringing overall predictors of population vaccine failure toward the null.

Limitations

Rates of low-level rotavirus infection, detectable by VP6 qRT-PCR, were high in non-rotavirus gastroenteritis controls. Rapid test negative infants who subsequently proved to be either EIA positive or had a viral load above the threshold associated with disease were excluded. However, the possibility remains that some of the infants included as non-rotavirus gastroenteritis controls were in fact rotavirus gastroenteritis cases. These infants could also be in the convalescent phase of a prior infection, or incubation phase of a new infection. Although this is a limitation of the study design, it should be noted that the rates of low level rotavirus infection in non-rotavirus gastroenteritis controls were similar to the rates of asymptomatic infection in community controls. In the context of high rates of low level rotavirus infection in this community, and given the high sensitivity of molecular methods of rotavirus detection, it would be logistically challenging to recruit large numbers of completely rotavirus negative controls. Nevertheless, it is accepted that misclassification of true rotavirus gastroenteritis cases (vaccine failures) as controls could risk bias towards the null.

Genotyping data was limited in some rotavirus cases, and a high proportion of asymptomatic infections, by technical difficulty in typing infections with low viral load. This is challenge which cannot be overcome within current molecular genotyping techniques, despite the nested PCR approach. Cases which could not be genotyped were excluded from the genotype-HBGA sub-analysis. This could limit the power of the analysis, due to reduced sample size, but avoids bias by the inclusion of cases and asymptomatic infections where genotype could not be determined. Genotype was not required for the primary outcome analysis of the case:control study, where the definition of clinical vaccine failure was severe rotavirus gastroenteritis confirmed by rotavirus ICT.

The limitations of HBGA phenotyping, particularly lower sensitivity/specificity of the Lewis ELISA assay, have been previously discussed (Chapter 3). However, it should be noted that in sensitivity analysis by FUT2 genotyping rather than phenotyping, the observed relationship between non-secretor phenotyping and odds of clinical vaccine failure was unchanged.

Summary

As shown in this and earlier chapters, rotavirus vaccine failure may be multifactorial, and interactions between predictors of vaccine failure and rotavirus infection complex. In the final chapter I will propose directions for future research which could further explore mechanisms of vaccine failure and test interventions to improve rotavirus vaccine effectiveness in Malawi and other low-income settings.

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6 CONCLUSIONS

6.1 OVERVIEW

The development and global roll-out of oral rotavirus vaccines has been a major scientific and public health success story, with a significant positive impact on child health worldwide. Despite this success, lower effectiveness of current globally-licensed vaccines in low-income, high-burden countries compared to higher-income countries remains a barrier to the ultimate aim of preventing deaths from rotavirus disease.

Using a representative study population in Malawi, a low-income country with a high burden of rotavirus disease, this thesis sought to explore possible contributory factors to rotavirus vaccine failure. My overall hypothesis was that reduced rotavirus vaccine response in Malawi is multifactorial, and is determined in part by HBGA phenotype, passively-acquired maternal RV-specific IgG, and oral polio vaccine response. My aim was to determine which of these three factors predict vaccine virus replication, host immune response and clinical protection in Malawian infants following oral administration of the G1[P8] human rotavirus vaccine (RV1). If found to contribute to reduced rotavirus vaccine response, targeted interventions, such as changes to scheduling or vaccine design, could be used to improve vaccine effectiveness.

Factors associated with vaccine virus replication and immune response were explored in a longitudinal cohort study, which recruited 293 infants. Factors associated with clinical protection were explored in a cross-sectional case-control study which screened 5990 gastroenteritis cases and recruited 120 severe rotavirus gastroenteritis cases (RV1 vaccine failures), 120 age-matched community controls and 95 age-matched severe non-rotavirus gastroenteritis controls.

This chapter will summarize my principal findings, reflect on limitations of this work, discuss policy implications, and outline implications and directions for future research.

6.2 SUMMARY OF PRINCIPAL FINDINGS

6.2.1 Oral vaccine response in Malawian infants

My study provided the first RV1 and OPV vaccine virus shedding data for Malawian infants, and the first immunogenicity data in Malawian infants for the current rotavirus vaccine schedule. Just over half of cohort infants had detectable rotavirus vaccine virus shedding by RT-PCR and 24% had rotavirus-specific IgA seroconversion following RV1 immunisation. Rates of seroconversion were much lower than in high income countries, but similar to other low-income settings. RV1 vaccine virus shedding, particularly following the 1st dose, predicted seroconversion.

In Chapters 3 and 4, complex patterns and interactions of rotavirus and poliovirus vaccine virus shedding and immune response were explored in detail. Reflections on this combined data within the context of current literature were used to develop a conceptual framework of oral vaccine response (Table 6-1).

Table 6-1: Conceptual framework: summary of sub-group characteristics

Sub-group	Shedding pattern	Immune response	Potential mechanism of inhibition	Predicted response to booster dose
Early responders	High shedding with short duration initial dose, low or no shedding later dose	Good response from initial dose	Optimal responders, no significant inhibition	Limited due to ceiling effects
Late responders	Low/no shedding initial dose, high shedding with short duration later dose	Poor response to initial dose, good response to later dose	Transient inhibition e.g. maternal antibody, OPV, co-infection	Good
Low shedders	Low/no shedding after each dose	Poor/no response	Persistent inhibition of vaccine virus replication e.g. unfavourable gut microbiome	Limited, any response may require multiple doses
Persistent shedders	High shedding with long duration after each dose	Poor/no response May eventually respond to multiple doses	Dysfunctional mucosal immune response e.g. environmental enteric dysfunction	Limited, any response may require multiple doses

Clearly, any framework of complex biological responses is an oversimplification. Vaccine response is a continuum, and the separation of groups is conceptual rather than absolute. However, this framework might help us explain different relationships between vaccine virus replication and immune response, and the factors which determine them, between different dose periods, between individuals, and between populations.

The proportion of infants who fall into each group will vary between populations. In high-income countries with high vaccine efficacy, we would expect a high proportion of early responders. Seroconversion would be high, but vaccine virus shedding would be relatively low, particularly after 2nd or subsequent doses. This is consistent with published data from high-income settings (Patel et al., 2013, Anderson, 2008).

In middle income or lower-middle income countries, such as those in Latin America, we would expect an overall majority of early and late responders, but a higher proportion of the poor response groups than in high-income countries. Within these countries, there would be sub-populations where poor response groups were in the majority, for example a higher rate of persistent shedders associated with the poor sanitation of peri-urban slums. In a mixed population like this there might be both moderate or high shedding and high seroconversion, but since some enteric vaccine virus shedding would be associated with a good immune response and some would not, the relationship between the two might tend toward the null. Again, this is consistent with published data (Patel et al., 2012, Becker-Dreps et al., 2017).

Finally, in low-income countries like Malawi, there may be a high proportion of both low shedders and persistent shedders, with a smaller proportion of early and late responders. In the overall population, this would predict a moderate or high proportion of vaccine virus shedding, but likely lower peak shedding levels, and low seroconversion. Strength of association between shedding and seroconversion would vary by dose and by the proportion of the population in each group. In populations with high proportions of persistent shedders, paradoxical shedding effects might be observed, such that higher enteric vaccine virus shedding is associated with reduced immunogenicity. Examples of each of these phenomena are shown throughout my data.

Large scale comparative cohort studies determining both effects on vaccine virus shedding and seroconversion in different, well characterized populations could provide further evidence for this conceptual framework and have implications for country-specific booster dosing strategies. This is discussed in detail below.

6.2.2 HBGA phenotype

The case-control study confirmed findings from previous *in vitro* and epidemiological studies that HBGA phenotype determines a strain-specific pattern of susceptibility to rotavirus gastroenteritis. In this Malawian population with high rotavirus diversity, secretor phenotype was strongly associated with P[8] rotavirus gastroenteritis, Lewis positive phenotype associated with P[4] rotavirus gastroenteritis, and Lewis negative phenotype associated with P[6] rotavirus gastroenteritis.

The cohort study tested the hypothesis that Lewis/secretor HBGA phenotype would predict G1P[8] vaccine virus replication and consequently immune response. There was some weak evidence that secretor HBGA phenotype was associated with increased rotavirus vaccine virus shedding following the 1st dose of vaccine, but there was no association observed with vaccine virus shedding overall and no association between HBGA phenotype and seroconversion. Since the inhibitory effect on shedding appears minimal, it may be that it is not sufficient to preclude an adequate mucosal immune response.

My initial hypothesis was that, as a result of reduced vaccine virus replication, non-secretor infants would have a reduced protective vaccine response, and consequently increased risk of clinical vaccine failure. Contrary to this hypothesis, odds of vaccine failure were over 60% lower in non-secretor infants. Reduced vaccine response in non-secretor infants is probably offset by reduced risk of P[8] and P[4] rotavirus infection, strains which accounted for over 60% of rotavirus gastroenteritis cases in this population.

In addition, 40% of non-secretor infants demonstrated G1P[8] vaccine virus shedding, and non-secretor phenotype was protective against P[8] gastroenteritis, but not against P[8] asymptomatic infection. This may suggest that non-secretor phenotype confers partial, but not absolute resistance to P[8] rotavirus infection. Non-secretor phenotype may protect against

P[8] rotavirus gastroenteritis, but not preclude vaccine virus replication or asymptomatic infection. Initial vaccine response may be further boosted by subsequent asymptomatic P[8] infection in non-secretor infants.

Similarly, my initial hypothesis was that, as a result of reduced vaccine virus replication, Lewis negative infants would have a reduced protective vaccine response, and consequently increased risk of clinical vaccine failure. Contrary to this hypothesis, 47% of Lewis negative infants demonstrated vaccine virus shedding. There was no association between Lewis phenotype and risk of clinical vaccine failure. This is likely because Lewis negative phenotype was protective against P[8] and P[4] rotavirus gastroenteritis, but increased risk of P[6] rotavirus gastroenteritis, bringing the overall risk of vaccine failure toward the null. In settings with a lower proportion of P[6] rotavirus, it is likely that Lewis negative phenotype, similarly to non-secretor phenotype, would be associated with a reduced risk of clinical vaccine failure.

The hypothesis that population differences in HBGA phenotype contribute to lower vaccine effectiveness in low-income countries depends on several assumptions.

Firstly, that there is a higher proportion of vaccine “resistant” HBGA phenotypes in low-income countries. While this is true for the Lewis negative phenotype in Malawi and some other African countries, it is not true for non-secretor phenotype, which occurs at a similar proportion in African and European populations.

Secondly, that reduced vaccine virus replication in infants with a “resistant” phenotype will prevent an effective immune response. There is some evidence to support this from other populations (Reyes et al., 2017, Kazi et al., 2017, Bucardo et al., 2018), however in this Malawian population, there was little evidence to support this assumption.

Finally, that this reduced vaccine response will outweigh the benefit of a “resistant” phenotype in protecting against the commonest strains of rotavirus gastroenteritis. This would result in increased risk of clinical vaccine failure in these infants. In Malawi, this is clearly not the case. Due to the protective association against P[8] and P[4] gastroenteritis, the relationship between Lewis negative phenotype and vaccine failure is equivocal and non-secretor phenotype is associated with reduced risk of clinical vaccine failure.

Based on the failure of this final and important assumption, it is highly unlikely that HBGA phenotype contributes to lower vaccine effectiveness in Malawi. It is also reasonable to assume that these results may be generalizable to other settings with similar HBGA distribution, force of infection and rotavirus diversity.

Understanding the relationship between HBGA phenotype and strain-specific susceptibility to rotavirus infection remains globally important to understanding viral epidemiology, viral/human co-evolution, and pathogenesis. However, it is unlikely that HBGA phenotype contributes significantly to population differences observed in rotavirus vaccine effectiveness between African and European countries.

6.2.3 Maternal antibody

There was some evidence to support my initial hypothesis that higher levels of maternal rotavirus-specific IgG antibody would inhibit rotavirus vaccine response. This is consistent with other recently published studies (Moon et al., 2016, Chilengi et al., 2016, Armah et al., 2016). Linear correlation was weak, with evidence instead for an inhibitory threshold effect, where only the highest maternal antibody levels were associated with reduced vaccine virus shedding and rotavirus-specific IgA response. This has important implications for vaccine scheduling, including timing of vaccine and the possibility of improving vaccine effectiveness through booster dosing. This will be discussed further below.

An alternative approach would include exploring whether lower vaccine responses due to maternal antibody inhibition could be overcome by prime-boost strategies, in a schedule combining oral rotavirus vaccines and newer non-replicating parenteral vaccines (Groome et al., 2017). Similar prime-boost strategies have proved effective in inducing mucosal immunity to poliovirus (Jafari et al., 2014).

6.2.4 Concurrent OPV

Contrary to the initial hypothesis that a robust response to OPV would be associated with reduced rotavirus vaccine response, the relationship between OPV and rotavirus vaccine virus shedding and immune response was more complex.

The inter-relationships determined between OPV and rotavirus vaccine responses were best understood within the conceptual framework (Table 6-1) and based on a new hypothesis that common factors result in similar patterns of response to both vaccines. Protective titres to type 3 poliovirus at 6 weeks were associated with increased likelihood of RV1 shedding in the first dose period, consistent with a group of infants who might be early responders to both oral vaccines. There was no overall association between OPV and rotavirus vaccine virus shedding in the 1st dose period, where the balance of early responders (negative association, low OPV shedding, high RV1 shedding), low shedders (positive association, low shedding both vaccines) and persistent shedders (positive association, high shedding both vaccines) may bring the association between the two toward the null. In contrast, OPV shedding in the 2nd dose period was associated with both increased likelihood of RV1 shedding, and reduced rotavirus seroconversion. This would be consistent with the effect of “persistent shedders”, infants with prolonged shedding but a dysfunctional immune response to both vaccines. By this stage, OPV and RV1 shedding would both be low in early responders and low shedders. In this way, OPV and RV1 shedding in the 2nd dose period would be positively, but not causally, associated.

Further evidence of the concept that there may be common vaccine response patterns, was provided by the positive association between serotype 3 poliovirus-specific protective titres at 12 weeks and rotavirus seroconversion. However, overall a higher proportion of infants attained poliovirus protective titres than achieved rotavirus seroconversion. Discordance in vaccine responses could be secondary to the additional benefit gained from a birth dose of OPV, or reflect a group of late responders to rotavirus vaccine secondary to transient inhibitory factors such as co-infection. Additionally, some inhibitory factors such as maternal antibody could have a greater impact on rotavirus than OPV immunogenicity.

Due to the limitations of an observational study design, the data do not completely exclude an inhibitory effect of concurrent OPV on rotavirus vaccine response. However, given the lack of quantitative correlation between OPV and RV1 vaccine virus shedding it is likely that any such inhibitory effect would be immune-mediated, rather than mediated through direct competitive inhibition of vaccine virus replication. This could be important in determining the likely impact of the global switch to inactivated parenteral polio vaccine, discussed further below.

6.2.5 Other factors predicting vaccine response

Compared to age-matched controls, infants with clinical rotavirus vaccine failure had much lower levels of rotavirus-specific IgA at presentation. Baseline rotavirus-specific IgA above the proposed protective level of 90U/mL was associated with a 75% decrease in the odds of vaccine failure. Following natural infection, infants with rotavirus vaccine failure were able to mount a robust rotavirus-specific IgA response. Within the case-control design, it was not possible to determine whether low rotavirus-specific IgA reflected primary vaccine failure or waning immunity. However, high convalescent IgA levels suggested that infants at the time of vaccine failure were capable of an effective mucosal immune response, at least to natural rotavirus infection. This has important implications for booster dosing strategies, discussed in detail below.

6.3 LIMITATIONS

My data must be considered within the limitations of the study. Specific limitations are discussed in detail within the relevant chapters. More general study limitations are as follows.

There are several limitations to my study design. Firstly, data were collected from only one population in a single geographic location. As discussed, patterns of rotavirus vaccine response may differ considerably between different populations, even between and within low-income countries. A multi-site study could have offered instructive comparison, but was not feasible within the logistic capacity of this doctoral project. To some extent, this limitation will be offset by plans to combine data from this study with the ongoing RoVi study, a multi-site study covering similar research questions and comparing mother-infant cohorts in Malawi, India and the UK (Sindhu et al., 2017).

Secondly, the key outcomes of vaccine virus replication, immune response and clinical protection were separated into a cohort and case control study. This meant that clinical protection could not be linked to factors at the time of immunisation, such as maternal antibody or OPV shedding, or to initial vaccine response. A single cohort study, with follow-up from birth to one year, would have been the ideal design to address all aspects of the research

question. However, given the relatively low frequency of severe rotavirus vaccine gastroenteritis (clinical vaccine failure) within the population, and the expected attrition, the sample size required for such a study would have amounted to several thousands. This was beyond the timescale and capacity of this doctoral project.

Finally, although adequately powered to address the primary research questions, numbers within the studies may be too small to address some of the secondary hypotheses. Sample size for OPV analysis was further limited by the global switch to bivalent OPV during the study period. This was unavoidable, but should be taken into consideration when interpreting results.

6.4 POLICY IMPLICATIONS

Data from this work will help inform vaccination strategies which may improve rotavirus vaccine effectiveness in Malawi and other low-income settings.

6.4.1 HBGA phenotype

The HBGA hypothesis implied that reduced vaccine response to P[8] based vaccines in non-secretors/Lewis negative infants might be avoided by use of non-P[8] based vaccines, such as the P[6] based RV3-BB (Bines et al., 2015) or P[11] based ROTAVAC® (Bhandari et al., 2014). As discussed, the study data suggest that HBGA phenotype does not significantly contribute to reduced rotavirus vaccine effectiveness in Malawi. Trial of newer vaccines in Malawi may be justified based on other advantages of these vaccines, for example cost, neonatal dosing or heat stability, but not on the basis of any hypothetical HBGA-related vaccine strain advantage.

6.4.2 Maternal antibody

The study data suggest a threshold inhibitory effect of high levels of maternal rotavirus-specific antibody on rotavirus vaccine response. The current vaccination schedule in Malawi of two doses of rotavirus vaccine given at 6 and 10 weeks in Malawi follows WHO guidance. Since maternal antibody declines exponentially from birth, delayed dosing of rotavirus vaccine could limit the inhibitory effect, by allowing time for maternal antibody to fall below the inhibitory threshold. However, delayed vaccination could risk delayed protection against rotavirus in

vulnerable infants. For logistic reasons, it is also preferable if dosing fits with other routine immunisations. Options for delayed or booster dosing strategies to offset the effect of maternal antibody inhibition and advantages and disadvantages of each are detailed in Table 6-2.

Table 6-2: Advantages and disadvantages of rotavirus delayed dosing regimes

Schedule	Advantages	Disadvantages
Two doses at 6 and 10 weeks	Current schedule Fits with 1 st and 2 nd dose of pentavalent vaccine and PCV	Evidence from study data of inhibition by maternal antibody
Two doses at 10 and 14 weeks	Schedule tested in Madhi et al. (2012) Fits with 2 nd and 3 rd dose of pentavalent vaccine and PCV May be more immunogenic than 6/10 week schedule (Steele et al., 2010)	Delays protection against rotavirus in younger infants
Three doses at 6, 10 and 14 weeks	Schedule tested in Madhi et al. (2012) Fits with 2 nd and 3 rd dose of pentavalent vaccine and PCV Trend toward higher immunogenicity than 10/14 week schedule in Madhi et al. (2012) Trend toward higher efficacy in 2 nd year of life in Malawi in (Cunliffe et al., 2012)	Higher cost than two-dose schedule
Two doses at 6 and 14 weeks	Fits with 1 st and 3 rd dose of pentavalent vaccine and PCV May provide protection in earlier infancy to early responders compared to a first dose at 10 weeks or later.	Untested schedule
Any above regime plus booster dose at 6 or 9 months	Booster dose fits with Vitamin A supplementation (6 months) or 1 st Measles rubella vaccine (9 months) Booster dose may also address waning immunity Evidence of modest benefit of booster dose at 9 months (Zaman et al., 2016)	Higher cost than two-dose schedule. In infants with poor response to initial schedule, delays protection to 6/9 months, the peak age of vaccine failure

The concept of booster dosing is also supported by the case-control data, where lower rotavirus-specific IgA predicts vaccine failure, but infants with rotavirus vaccine failure generate effective convalescent IgA response. This suggests booster dosing in later infancy could have the potential to similarly stimulate a more protective mucosal immune response.

The Malawi Ministry of Health should consider including trials and other research to determine optimal rotavirus vaccination strategies within the National Health Research Agenda. Possible research designs are discussed further below.

Alternatively, new neonatal rotavirus vaccines are reportedly not subject to inhibition by maternal antibody. A Phase IIb trial of RV3-BB will begin in Malawi in 2018. Immunogenicity results from this study should be valuable in determining the potential of this new vaccine. Trials of other new rotavirus vaccines should similarly be supported.

6.4.3 Concurrent OPV

Evidence from several studies suggest an inhibitory effect of concurrent OPV on rotavirus vaccine response, but data are limited and not conclusive. In Malawi, as part of the Global Polio Eradication Initiative, the introduction of inactivated parenteral polio vaccine to the national schedule is imminent. An initial combined schedule of IPV and bivalent OPV will likely switch to IPV only within the next five to ten years. The switch to IPV may well be associated with improved rotavirus vaccine responses, and data from this thesis should not detract from current plans by the Malawian Ministry of Health for its introduction.

However, the mechanisms of OPV inhibition of rotavirus vaccine response are not well described. My study found no evidence that OPV vaccine shedding directly inhibited rotavirus vaccine shedding, suggesting that any inhibitory effect may be immune-mediated. The indirect effects of OPV on the infant microbiome in low-income settings are unknown, but Sabin vaccine viruses are known to inhibit other enteroviruses, which in turn have been shown to inhibit rotavirus vaccine responses (Taniuchi et al., 2016). Overall, the impact of the switch to IPV on rotavirus vaccine responses in Malawi and similar countries may be unpredictable. Further research at the time of the switch to determine the impact on rotavirus vaccine response should be supported as a priority by the Malawian Ministry of Health.

6.5 RESEARCH IMPLICATIONS AND FUTURE DIRECTIONS

6.5.1 Research implications

One of the key challenges in rotavirus vaccine trials has been the lack of confirmed correlates of protection. Early correlates or surrogates of protection clearly linked to clinical outcomes could allow better comparison of new vaccines in phase II studies. This is particularly important now rotavirus vaccines have been widely introduced and placebo-controlled studies of new vaccines present an ethical challenge.

My study provides new data on both vaccine virus shedding and rotavirus-specific IgA as markers of rotavirus vaccine take which may inform future studies seeking to address this problem.

My data on post-immunisation rotavirus vaccine virus shedding, using sensitive and specific molecular methods in a large cohort with serial samples following both vaccine doses, represents one of the most detailed studies of post-immunisation rotavirus shedding to date. The observed correlation of vaccine virus shedding following the first dose with immune response supports the concept that vaccine virus shedding is a valid measure of vaccine take. My serial shedding data also identifies day six post-immunisation as the optimum time-point to measure vaccine virus shedding, predictive of shedding overall. This should be useful in designing future studies in similar populations, to limit the cost and logistical challenge of serial sampling. Measurement of faecal vaccine virus shedding has significant ethical, logistical and cost advantages as a measure of vaccine take compared to measurement of rotavirus-specific IgA. Confirmation of vaccine virus shedding as a surrogate of protection would require linking shedding with clinical protection in large studies. This could be achieved by including vaccine virus shedding in a large-scale rotavirus vaccine trial.

Rotavirus-specific IgA is the best currently available correlate of protection for rotavirus vaccines at population level, but data linking rotavirus-specific IgA levels to clinical protection at individual level have been limited and no threshold of protection has been established. In contrast to some previous studies (Premkumar et al., 2014), low rotavirus specific-IgA at time of

presentation was clearly associated with increased risk of vaccine failure in infants in Malawi. Rotavirus-specific IgA above the proposed “protective” level of 90U/mL was associated with significantly reduced risk of vaccine failure. These data support the concept that rotavirus-specific IgA represents a useful correlate of protection for rotavirus, and may be particularly relevant to studies of booster strategies, as discussed below.

6.5.2 Future directions

The data and concepts developed in this thesis lead to two interlinked themes of further research within which several new research questions may be addressed.

Theme 1: Understanding population patterns of rotavirus vaccine response.

Patterns of rotavirus vaccine responses are complex. Based on my data, and wider literature, I have proposed a conceptual framework which categorises infants within different sub-groups of vaccine response. The balance of each sub-group within a population may predict overall vaccine response, relationship to inhibitory factors, and response to intervention. Both further detailed analysis of available data, and new data in diverse populations is required to confirm this concept, better characterize sub-groups and potentially identify new patterns. Data analysis could take several analytic approaches including mathematical modelling of population level vaccine response patterns; principal components analysis or factor analysis to explore patterns within populations; or Markov models to explore conditional probabilities of initial vaccine response patterns predicting response to future doses.

The question of whether there is significant waning of vaccine-generated rotavirus protective immunity in the first year, particularly in low-income countries, remains unanswered. This is a complex question to address, since the dynamics of immune response to rotavirus in infancy are also influenced by ongoing exposure to natural infection, and the effects of maternal antibody. There is an urgent need to understand the dynamics of post-immunisation response to potentially explain population differences in vaccine efficacy and determine the likely utility of booster dosing strategies.

Better understanding of patterns of rotavirus vaccine response within populations could lead to more targeted interventions including optimization of vaccine schedules. The WHO recommendation for universal rotavirus immunisation was an important step in the global introduction of rotavirus vaccines. However, it is now important to move away from the concept that “one schedule fits all”. Different factors may contribute to different patterns of poor vaccine response in different settings – strategies which fail to improve vaccine response in Bangladesh may be successful in Malawi. More detailed information on different modes of vaccine failure and the relative frequency of each within a population could help predict which interventions will work and where. In short, future research into rotavirus vaccine responses may need to take a conceptual step backwards. Rather than focusing on the question “Why do rotavirus vaccines fail in low-income countries?” it may be more productive in the long-term to first ask, “How do rotavirus vaccines fail in low-income countries?”. Large-scale, multi-country cohort studies would provide the best data.

Theme 2: Optimising rotavirus vaccine schedules

The clear need to establish a stronger foundation of our understanding of rotavirus vaccine responses must be balanced against the immediate need to improve vaccine effectiveness in low-income countries to reduce child deaths from rotavirus. Although development of new vaccines, including parenteral vaccines, may ultimately improve outcomes, this will take considerable time with no guarantee of success. As discussed above, my data support the concept that booster dosing may be a promising strategy to improve rotavirus vaccine response. Booster dosing is also one of the simpler interventions to implement quickly and at national scale. The key question is optimal timing, which may depend on several factors including dynamics of the rotavirus immune response, force of infection, and logistic considerations, and as discussed may vary between populations. Timing would ideally be determined by a randomized controlled trial, with one or more booster dosing regimens compared to the standard two-dose schedule.

Theme 1 would be best addressed by multi-country birth cohort studies, and Theme 2 by a randomized controlled trial. Potentially, these studies could be integrated. However, these

studies would require sample sizes in the thousands, would take several years to complete, would be logistically challenging and would be very expensive. Pilot data which could inform the design of such studies, particularly the timing of booster dose study arms, could lead to more efficient study design and improve the likelihood of successful intervention.

A proposed study: Rota-Challenge

Such pilot data might be provided by a rotavirus vaccine challenge study. The vaccine challenge approach has been well-established in testing responses to oral poliovirus vaccines (Hird and Grassly, 2012). In my proposed challenge study, infants who had previously received two doses of RV1 at 6 and 10 weeks, would be recruited in stratified age groups from 14 weeks to one year. Infants would then be randomized to receive either a further dose of RV1 or placebo. Vaccine virus and wild-type rotavirus faecal shedding would be measured at baseline, at six days and at four weeks post-dose. Rotavirus-specific IgA and IgG would be measured at baseline, and at four weeks post dose (Figure 6-1).

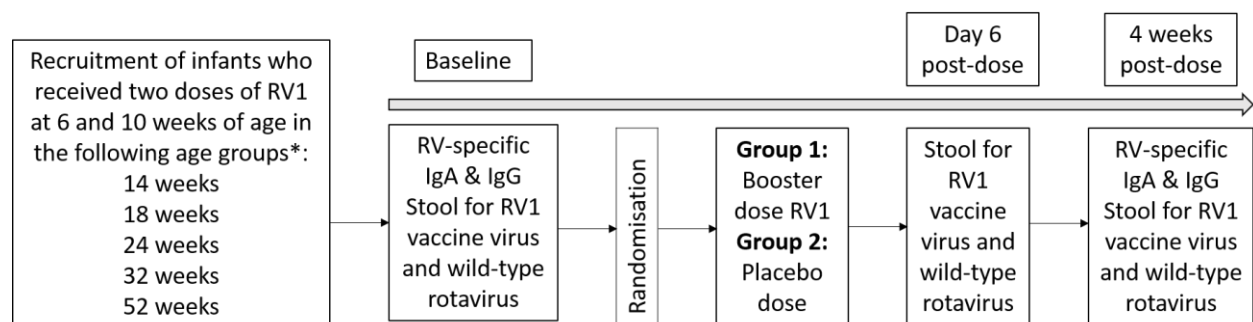


Figure 6-1: Proposed rotavirus vaccine challenge study

**Age groups are chosen to relate to time-points which would complement the existing immunisation schedule. Additional age groups would add useful data, but at additional cost.*

The placebo group would provide cross-sectional data on both rotavirus-specific IgA and IgG at different ages in the first year of life. This data could be used to model the dynamics of rotavirus-specific IgA and IgG over the first year at population level. If rotavirus immunity does wane over time, this approach could identify the population nadir of RV-specific IgA and target timing of a booster dose. Although a longitudinal cohort design would be preferable to model dynamic changes in individuals over time, a cross-sectional design avoids the ethical and logistic challenges of serial blood sampling of infants and provides useful population data. Similarly, the

placebo group would provide data on persistent shedding of vaccine virus and prevalence of asymptomatic wild-type rotavirus.

The challenge group would provide similar cross-sectional baseline data to the placebo group. In addition, the challenge group would provide data on both vaccine virus shedding and immune response to a further dose of RV1 at different ages and at different baseline levels of rotavirus-specific IgG and IgA. This could address the following research questions:

- Does booster dosing of RV1 result in a seropositive RV-specific IgA response (IgA>20U/mL) in infants seronegative at baseline? Or seroconversion (four-fold rise) in infants seropositive at baseline?
- Does RV-specific IgA at baseline predict vaccine virus shedding post-booster dose?
- Is RV-specific IgA immune response to booster dosing of RV1 inhibited by baseline RV-specific IgG? In infants under 6 months this might reflect maternal antibody, in older infants prior rotavirus exposure.
- Does RV-specific IgA response or vaccine virus shedding following booster dosing vary by age?

This pragmatic study design clearly has limitations, but also has a number of practical advantages. The cross-sectional design means the study could be completed within a much shorter timescale than a longitudinal cohort design. Although sample size required would still be large, to allow comparison between different age groups, attrition would be limited by the short follow-up period. Costs would be significantly less than a cohort or multi-arm randomized controlled trial. Data from this pilot study could then predict the likely success of booster dosing as an intervention and inform the design of any subsequent trial.

6.6 CONCLUSION

Despite the global success of rotavirus vaccines, many children in low-income countries like Malawi remain vulnerable to this preventable disease. It is essential to maintain the momentum of rotavirus research to optimize current vaccine schedules, design new and more effective vaccines and improve clinical outcomes for children worldwide.

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